PHYSIOLOGICAL, BIOCHEMICAL, AND MOLECULAR

MECHANISMS ASSOCIATED WITH DROUGHT TOLERANCE IN

AGROSTIS SPECIES

By

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ABSTRACT OF THE DISSERTATION

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Improvement of the drought stress tolerance of plants is necessary due to the widespread incidence of drought damage to crop species. Turfgrasses are susceptible to drought damage and may exhibit symptoms of cellular water loss such as wilting, cessation of growth, and other cellular damages resulting in leaf and root senescence. Creeping bentgrass (*Agrostis stolonifera* L.) is a high value, drought sensitive turfgrass crop species. The main goals of the research described in this thesis were to evaluate mechanisms responsible for drought tolerance in turfgrasses by evaluating whole-plant, cellular, proteomic, metabolomic, genetic, and genomic regions associated with drought defense responses.

Part I will focus on how differential hormonal regulation may affect the drought defense responses in turfgrasses. Plant hormones such as cytokinins (CK) are signaling molecules controlling gene expression and the activity of various biochemical pathways. Differential drought-induced regulation of plant hormones is a primary response to prevent cellular desiccation. Drought injury symptoms have been associated with an inhibition in CK synthesis and maintenance of endogenous CK is associated with alleviation of drought damage. Thus, specific

objectives related to the effect of elevated CK content in creeping bentgrass during drought stress on 1) whole-plant physiology 2) proteomic 3) metabolic and 4) genetic responses were evaluated. Elevated CK content in the creeping bentgrass plants was achieved by drought induced expression of an *ipt* transgene encoding the enzyme adenine isopentenyltransferase promoting CK synthesis. The results showed significant modifications of the gene, protein, and metabolite profiles were caused by elevated CK, particularly changes related to energy production, metabolism, and stress defense.

Part II will focus on the identification of genomic regions associated with drought tolerance known as quantitative trait loci (QTL). QTL are large genomic regions that are associated with molecular markers and specific plant phenotypes that can be used in plant breeding strategies. Knowledge of the location of QTLs can help breeders screen large quantities of germplasm for complex traits such as drought tolerance. QTLs for important drought tolerance traits such as relative water content, cellular membrane stability, indexes of turf quality, leaf area, and chlorophyll content were found.

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LITERATURE REVIEW

Introduction

The grasses are a large family of over 9000 C3 and C4 monocot plant

species within the *Poaceae* family (USDA, NRCS. 2010). They are extremely

important to our society in many ways since they play a major role in shaping the

world ecologically, economically, and socially. Ecologically, natural grasslands

contribute to the health and well-being of our ecosystem by providing a home to

many animal species, reducing pollution by trapping run-off, preventing land

erosion, and sequestering the greenhouse gas carbon dioxide. In agriculture and

industry, they are a food source for the grazing animals in pasture lands and are

grown as high value crops for use as natural fibers, bioenergy crops, or sod.

The grasses with perhaps the most social impact on our lives are

turfgrasses. Turfgrasses are a subset of the grasses, which have evolved and have

been bred by man to tolerate mowing and traffic. This has allowed turfgrasses to

be used as a ground cover in parks, home lawns, playing fields, and golf courses.

Plant breeders have targeted certain grass species that have evolved over millions

of years due to their co-existence with animal grazing due to their ground cover

and aesthetic appeal qualities, allowing them to persist in a mowed, uniform

canopy. Historic plant breeders recognized that these characteristics are desirable

in various aspects of society and have therefore done extensive research to

identify key species and traits within the grasses to optimize seed and sod

production, cultural management practices, and optimal turfgrass species selection for a given climate. In cultured, relatively high input settings, turfgrasses serve numerous functions such as providing a cushioned, uniform functional surface for human recreation and aesthetic appeal. Turfgrasses may also be utilized in low input and/or low maintenance settings, such as on road sides, edges of water ways, or in preserved land, where they may act as barriers to reduce pollution and run-off, reduce wind erosion of the underlying soil, preventing dust, and serving as havens for numerous insects and wildlife (Turgeon, 2008).

The versatility and high demand for turfgrasses has allowed the turfgrass industry to exhibit extensive economic impact (Haydu et al., 2006). However, the industry faces two main challenges: creating optimum growing conditions to appease both humans and the grass. Pleasing the people that are most intimately linked with turfgrasses such as homeowners and athletes requires turfgrass managers to maintain healthy and functional turf at high standards often under environmentally and economically limited conditions. This has lead to critics of turfgrass management, which is labor intensive and can require a heavy regimen of fertilizer and pesticide input. Critics say they are point sources for run-off of pesticides, fertilizers, and other chemicals used for maintenance, they require much input in the form of energy which ultimately utilizes our fossil fuel resources, and that they are superfluous, especially in times of economic crisis (Steinberg, 2006). The second challenge, pleasing the grass, requires much research on defining optimum cultural practices, turfgrass pathology, and grass physiology. Perhaps the most successful strategy to pleasing both the grass and

humans is utilization of the right grass germplam for the environment, to which understanding the physiology of each grass becomes important. Starting with a grass suited for an environment and one that is able to tolerate environmental stresses is the ultimate method to reduce inputs, costs, and criticisms.

The most prevalent abiotic stress to plant growth is drought stress and water for irrigation is the most limited resource world-wide (Khush, 1999). Despite the great versatility and availability of the grass species used as turfgrasses, their drought resistance is relatively low compared to other crop plants. In addition, drought is a complex abiotic stress that impacts all plant organs and cell types and requires multiple resistance mechanisms within the plants. Thus, the focus of this dissertation is on multiple methods of identifying and improving drought resistance mechanisms of a commonly used relatively drought sensitive turfgrass, creeping bentgrass (*Agrostis stolonifera* L). The current chapter provides a review of recent literature important to the understanding of the drought response and will conclude with the specific goals of this dissertation.

Importance of Creeping Bentgrass

Creeping bentgrass is naturalized to most parts of the world but the varieties used today as turfgrasses were imported to the US from its native Eurasia in a seed mixture that was termed "South German mixed bent." After many decades of production, growth, and selection from the mix, *Agrostis stolonifera* became the primary bentgrass species desired and grown (MacBryde,

2005). Today, creeping bentgrass is a high value crop, whether it is grown for seed, sod, or strictly as a turf, contributing to a great percentage of the multi-billion dollar worldwide turf industry. For example, sod production revenues from the largest sod producing states such as Rhode Island, New Jersey, and Michigan have reached over $15 million annually (Siligato, 1999). A large percentage of the seed and sod produced is creeping bentgrass since it is frequently used on golf course greens, fairways, and athletic fields. However, most turf managers grow it from seed or plugs arising from abiotic propagation from stolons. These growth methods are viable options because it often establishes and spreads quickly due to its stoloniferous growth habit and tolerates low cutting heights (Emmons 1995) Creeping bentgrass can also be used as a forage grass or for home lawns, however the latter is not common due to the high input and care requirements relative to other grasses such as Kentucky bluegrass (*Poa pratensis* L.). Furthermore, the interest in use of creeping bentgrass is increasing from past levels due to its productive growth in many areas. For example, a recent case study in the US revealed that many golf courses are switching to growing creeping bentgrass on golf course greens and fairways from other species due to lower disease incidences and input costs (Vermeulen, 2000). In addition to its economic importance and potential for future success, creeping bentgrass serves as a great model plant species in research within several disciplines. For instance, the *Agrostis* genera is intriguing genetically due to it highly complex ploidy levels and interspecific hybridization abilities. In addition, creeping bentgrass and other *Agrostis* species are known for being tolerant of metal contamination and having a

variation in range of abiotic and biotic stress resistances thereby making potential genetic studies and research topics on *Agrostis* species widespread (MacBryde, 2005).

Genetic Attributes and Cultural Practices Contributing to Drought Sensitivity

Genetic factors controlling the growth habit and morphology of creeping bentgrass contribute to its sensitivity to drought stress. Plants typically utilize various resistance mechanisms including those categorized as escape, avoidance, or tolerance responses to drought stress (Huang, 2006). Escape is typically characterized as a state of dormancy or a necrosis by programmed cell death of cellular tissues except those that are required for regeneration or regrowth upon a change of season or alleviation of a stress such as renewal of available water after prolonged drought conditions. Drought avoidance responses are a group of mechanisms, such as deep rooting (Cairns *et al.*, 2004) or leaf curling, to prevent, reduce, or delay cellular dehydration (Pessarakli, 2008). Creeping bentgrass actively alters leaf morphology in order to reduce water loss by leaf curling and folding to reduce leaf surface area for water loss but lacks other genetic traits governing drought avoidance such as the presence of morphological characters such as leaf hairs, low stomatal density, thick waxy cuticles, trichomes, or sunken stomata (Ramanjulu and Bartels, 2002).Under natural conditions, creeping bentgrass exhibits some avoidance and escape mechanisms primarily in terms of cessation of growth, promotion of root growth, leaf rolling, and stimulation of

rooting (Fry and Huang, 2004). However, the ability of creeping bentgrass to escape and avoid drought stress under strict turfgrass management conditions are not typically viable mechanisms due to the requirements of functionality and cultural practices used in most turfgrass areas.

Therefore, the sensitivity of creeping bentgrass to drought stress is due to both genetic factors and typical turf management practices that limit drought escape and drought avoidance mechanisms. For instance, on a golf course green, creeping bentgrass is often mown at heights as low as 0.3 cm. Low mowing heights may impede a plant's ability to exhibit some drought avoidance or escape mechanisms of both above and below ground plant parts. For example, the reduction in leaf length and increase in leaf width may affect leaf curling, the canopy as a whole may be less humid thereby preventing the buildup of high leaf boundary layer humidity otherwise typical of plant canopies, and shoot succulence increases but typically at the expense of other cellular traits such as a tough thick cell wall (Fry and Huang, 2004). Below ground, the plant naturally finds a balance of the root:shoot ratio and will therefore have a smaller root system reducing the potential for water uptake from deeper in the soil profile. Limitations on root biomass will not only limit the discovery of water sources deeper in the soil profile but will also restrict hydraulic lift that brings water deeper in the soil profile to roots closer to the soil surface (Huang 1999). In addition to limitations on mechanisms related to water use and uptake, various turf management practices may limit other processes important to stress avoidance or escape such as reduced photosynthesis and respiration for energy

production and reduced biomass of storage tissues available for carbohydrate reserves. Inadequate carbohydrate reserves and available energy may severely limit the efficacy of stress defense pathways and are typically reduced with a reduction in the size of plant organs (leaves, roots, and storage organs such as crowns and rhizomes). Therefore, since escape or avoidance characteristics may be limited by turf management practices, the research goals within this dissertation were to evaluate, identify, and explore drought tolerance traits in creeping bentgrass maintained as a turfgrass. The ultimate goal of this type of research is to elucidate drought tolerance traits and mechanisms that will be beneficial for future use by plant breeders.

Drought Stress Perception

In order for drought resistance or tolerance mechanisms to be activated, plant cells must sense an above or below ground incidence of an imbalance between water loss and water availability and then convert that perception into a cellular stress signal. As sessile organisms, plants have evolved a complex signaling network that conveys stress messages throughout the plant via multiple primary and secondary signaling transduction pathways. These pathways consist of various types of signaling molecules since a combination of hormone signals coupled with the accumulation of other metabolic compounds such as reactive oxygen species, proteins, and other osmolytes are often required in order for changes in gene expression to occur. These compounds may be either actively produced by the plant or accumulate as a result of cellular damage (Ramanjulu

and Bartels, 2002). The signaling cascades that occur may be either the cause of and/or are in response to the perception of drought stress to actively initiate further downstream changes in gene expression leading to plant drought resistance and are initiated by plant hormone signaling pathways.

Changes in endogenous hormonal content primarily occur in order to activate drought tolerance mechanisms described below. The biosynthesis, repression, and cellular targeting of hormones may change depending on the hormone type and its function. Major plant hormones that are important in the drought response are ABA, cytokinins (CK), Jasomonic acid, ethylene, and others. Drought stress is thought to be perceived as a hydraulic pull caused by soil to plant gradient of pressure due to soil drying. When the hydraulic pull is sensed, the result is a shift in the concentration of the signal hormones abscisic acid ABA (Davies and Zhang, 1991; Raghavendra, 2010). ABA typically increases in concentration in order to convey the drought stress signals (Zeevart et al., 1988) wheras other homones such as CKs may be reduced by downregulation of gene expression, degraded by oxidase enzymes actively, or due to stress damage (Bray, 1993). These changes are complex and dynamic since hormone concentration may act independently to confer a signal or it may act in conjunction with other hormones and/or with other signals. Furthermore, the endogenous concentration of a given hormone may be influenced by the duration and severity of drought stress and may differ in the different plant organs. For instance, Sharp *et al.* (2002) has shown that hormones working in conjunction with each other is exemplified by the indirect role of ABA in water stress signaling by inhibiting the

synthesis of ethylene (Sharp and LeNoble, 2002; Chaves and Oliveira, 2004). ABA-dependent and ABA-independent signaling pathways are used to elicit a response to drought and a rapid accumulation of ABA has been correlated with enhanced drought resistance (Li et al., 2000). In studies of the highly drought tolerant resurrection plants (*Craterostigma wilmsii*), ABA concentrations were shown to be the most highly affected hormone in response to drought stress (Vicre *et al.*, 2004). ABA and other hormonal signaling pathways lead to major chages in plant growth, defense responses, and major drought tolerance mechanisms.

Drought Stress Signaling

Plant survival of stressful conditions such as drought is governed by the capacity for quick recognition of the stress and the rate of induction of protective mechanisms. The rapid closure of stomata is crucial for plant survival in drying environments. Stomatal closure is often described as the first line of defense since its response to water deficit is much quicker than other physiological changes. Stomatal closure reduces transpirational water loss and reduces water consumption. It is believed that when roots are exposed to drought stress a chemical signal is transported to shoots, inducing stomatal closure. The involvement of root-to-shoot signaling in regulating stomatal behavior has been found to play important roles in plant tolerance to drought stress (Quarrie, 1989; Wilkinson and Davies, 2002).

Abscisic acid (ABA) is considered as the primary chemical signal translocated from roots to shoots causing stomatal closure in response to soil drying (Blackman and Davies, 1985; Zhang and Davies, 1989; Davies *et al.*, 2002). Increases in ABA concentrations in guard cells triggers a signal transduction cascade, including promoting the efflux of potassium ions from guard cells, which causes reduction in turgor pressure of guard cells and ultimately the closure of stomata (Leckie *et al.*, 1998). ABA also mediates cytosolic Ca^{2+} levels and triggers Ca^{2+} mediated pathways by regulating movements through Ca^{2+} channels. Cytosolic Ca^{2+} then transmits the signal to protein sensors such as calmodulin, Ca^{2+}-dependent protein kinases (CDPK), and phosphatases, which play roles in ion channel regulation (Luan, 2002). Calcium binding proteins such as calcineurin B-like (CBL) proteins are also important in calcium signaling pathways and are thought to contribute to early stress-related transcription factor regulation (Albrecht *et al.*, 2003).

Transcription factors are the stress response elements that perhaps have the most potential for enhancing tolerance mechanisms for multiple stresses. In *Arabidopsis*, transcription factor families ERF/AP2, bZIP/HD-ZIP, Myb, WRKY, and several classes of zinc- finger proteins, each containing a distinct type of DNA binding domain, have all been characterized. These transcription factors bind the stress-responsive cis-elements and activate the expression of target genes (Yu *et al.*, 2005). The target genes have end-products for various key players in the physiological response such as ABA.

In addition to the drought tolerance exhibited by the ABFs discussed above, Kim *et al.* (2004) observed multiple stress tolerance in transgenic plants over-expressing ABF3. Transgenic lines were tolerant of low-temperatures, heat, and oxidative damage. Similarly, overexpression of the pepper transcription factor CaPF1 in transgenic Virginia pine (*Pinus virginiana* Mill.) conferred multiple stress tolerance by increasing plant oxidative stress defenses (Tang *et al.*, 2005). Other cis-acting elements that have been the topic of much research are dehydration responsive elements and ABREs since many stress-inducible genes contain these elements in their promoter regions. Amongst others, NAC transcription factors, bZIP proteins such as TRAB1, and MYB activators bind to these regions and have been shown to upregulate certain stress responsive genes to enhance plant defenses (Tran *et al.*, 2004; Narusaka *et al.*, 2003; Kagaya *et al.*, 2002). For example, Suzuki *et al.* (2005) have reported that constitutive expression of the stress-response transcriptional coactivator multiprotein bridging factor 1c (MBF1c) in *Arabidopsis* enhanced the tolerance of transgenic plants to heat or osmotic stress alone, as well as the combination of both stresses. Most importantly, the expression of MBF1c augmented the accumulation of a number of defense transcripts in response to heat stress via the ethylene-response signal transduction pathway (Suzuki *et al.*, 2005).

In addition to signaling causing stomatal closure and the growth reductions caused by decrease intracellular CO_2, Other proteins respond to drought stress and ABA content by playing a role in signaling plant leaves to decrease growth by inhibition of cellular division and expansion. For instance, a

reduction in leaf expansion has been associated with hormonal signaling causing membrane bound ATPases, LEA proteins, expansins, phospholipases, and peroxidases to become differentially activated. ATPases may become deactivated by drought stress, in order to reduce decreases in cross membrane pH often associated with stress (Chaves et al., 2003). Expansins and LEA proteins may increase in response to drought stress to maintain the cell wall structure and degree of extensibility. These proteins may actively cause signaling cascade changes. Maintenance of expansins, LEA proteins, and others such as xyloglucan endotransglycosylases have also been associated with conveying increased drought tolerance and avoidance (Jones and McQueen-Mason, 2004; Sharp *et al.*, 2004). Mitogen-activated protein kinases (MAPKs) are involved in plant signaling in response to drought stress (Yu *et al.*, 2005b). Important proteins such as these involved in drought signaling, in conjunction with other signals such as osmotic regulation, lead to crossmembrane extracellular signaling cascades to reduce leaf growth and activate other drought tolerance mechanisms.

Free reactive oxygen species (ROS) accumulation has also been shown to be a stress signaling mechanism in response to drought. ROS accumulation caused by stress is both detrimental and beneficial to plant survival due to the damage they cause at high levels and their role in stress signaling, respectively. ROS, particularly H_2O_2, are primarily produced due to the enhanced enzymatic activities of plasma-membrane-bound NADPH oxidases, cell-wall-bound peroxidases, and amine oxidases in the apoplast during the stress response. They are involved in signaling various defense mechanisms such as stomatal closure

and root elongation, often by interaction with Ca^{2+} channels and other signaling proteins such as MAPKs (Laloi *et al.*, 2004). Once the stress signal is perceived it is necessary for plants to remove these harmful byproducts during recovery.

Drought Tolerance Mechanisms

After stress perception and signaling, drought tolerance mechanisms are activated. These mechanisms allow the plant to maintain physiological functioning under limited water conditions and the extent to which these mechanisms are effective define the relative drought tolerance of the plant. The definition of drought tolerance may be expressed differently based on the performance requirements of various crop species, since it may be evaluated primarily based on traits such as yield or biomass accumulation (Blum, 2005). For turfgrasses, plants that are able to maintain green color, uniformity of growth, and functionality as a playing surface under stress conditions are considered the most drought tolerant (Turgeon, 2008). Since drought tolerance of turfgrasses requires a variety of phenotypic traits to be expressed, involving multiple physiological and biochemical pathways, it is considered a complex, quantitative trait. Creeping bentgrass is a cool season grass species utilizing the C3 metabolic pathway, which is generally less tolerant of stresses such as heat and drought relative to warm season C4 species. Major pathways such as those involved in stress signaling, energy production, carbohydrate storage and metabolism, protein synthesis, and many others may all be adversely affected by drought stress and may differ in the response to drought stress. Each pathway also has key

components that may be involved in conferring drought tolerance by regulation of its own pathway or by providing crosstalk to regulate other pathways. Due to this complexity, the remainder of the introduction will focus on drought tolerance mechansims and the effects of drought stress on major metabolic pathways and several key regulators of the drought tolerance responses important to creeping bentgrass and other major turfgrasses

Stomatal Aperture Regulation

One of the primary defenses against dehydration of plant leaves during drought stress is by efficient regulation of guard cell turgor pressure to quickly regulate stomatal apperatures. Stomatal aperture is regulated by turgor pressure fluctuations determined by the osmotic status of the guard cells surrounding the stomatal pore (Li et al., 2000). These osmotic functions are regulated by ion channels primarily controlled by ABA. ABA elicits stomatal closure during drought stress by inactivating ion channels to prevent movement of osmolytes such as potassium and chloride ions and sugars such as malate and sucrose into guard cells (Wang et al., 2001). Stomatal closure is considered a drought tolereance mechanism because it will limit water loss through transpiration. However, morphological and environmental factors may predict how quickly and how long stomata need to be closed in a certain species and therefore indicate its capacity for drought tolerance. In C_3 turfgrass species in particular, a delicate balance exists between the degree of stomatal aperture and drought tolerance. If stomata need to remain closed too early or too long, the diffusion of CO_2 for photosynthesis and carbohydrate production in leaves will be reduced and will

concomitantly cause increases in O_2 concentration. These changes will lead to reduced drought tolerance due to inadequate carbohydrate and energy production and other metabolic damages such as from ROS accumulation (Nilsen and Orcutt, 1996). The effects of drought and stomatal closure on photosynthesis and reactive oxygen species are discussed in more detail below.

Maintenance of Carbon Metabolism

Maintenance of a balance of carbohydrate anabolism and catabolism under stress conditions for continued growth and stress defense under drought conditions is largely due to the rates of photosynthesis, respiration, and sugar mobilization. During drought stress, stomatal and non-stomatal limitations of photosynthesis may occur. Stomatal limitation of carbon fixation is due to actively regulated stomatal closure to prevent water lost by transpiration. Reducing transpiration in this manner prevents movement of carbon dioxide through stomatal apertures. This decline in available carbon dioxide slows the flux through carbon fixation pathways. The photosynthetic rate may quickly recover following rehydration if the photosynthetic apparatus is not permanently damaged (Foyer *et al.*, 1998). C3 plants such as creeping bentgrass are particularly sensitive to stomatal limitations to photosynthesis during conditions when water is limited (Hu et al., 2010). Transient or permanent damage to photosynthetic machinery typically caused by prolonged drought stress is known as non-stomatal limitation to carbon uptake (Parry et al., 2002). Both stomatal and non-stomatal limitations may cause a reduction in photosynthetic rates typically observed as reduced carboxylation efficiency of RUBP, slow rates of RUBP

regeneration, loss of photosynthetic enzyme activity of enzymes involved in the dark and light reactions, a loss of photosystem health, and low photochemical efficiency (Lawlor and Cornic, 2002).

Specific reductions in enzymatic activity or protein content are a major cause of reduction in biochemical pathways such as photosynthesis under drought stress. The sensitivity of various enzymes is genetically controlled and is related to other factors such as the presence of antioxidants, molecular chaperones, or the stability of other organelles and membrane structures, as discussed in more detail below. Drought stress typically causes a reduction in the content and functionality of major enzymes such as RuBisCo and GAPDH; however, increased expression of some enzymes involved in major metabolic pathways has also been detected (Penuelas et al., 2005). While reduction in photosynthesis under drought stress may be due to both stomatal and non-stomatal (metabolic) limitations, stomatal limitation of CO_2 supply may be more important during the early phase of drought and non-stomatal impairment becomes more pronounced following prolonged or severe drought stress. Similar to plants under heat stress, under severe water limitation net photosynthetic rate may decrease due to the decrease in the activity of Rubisco and the abundance of Rubisco small subunit (rbcS) transcripts (Penuelas *et al.*, 2005). In tomato *(Solanum lycopersicum)*, the mechanism of reduced activity is thought to be due to the presence of intracellular inhibitors such as CA1P and 'daytime inhibitor' (Parry *et al.*, 2002). These inhibitors are thought to bind to Rubisco in unstressed conditions to prevent the destruction of inactive Rubisco by proteases. Simultaneously, the same

experiment was done on wheat (*Triticum aestivum* L.), however, the results did

not conclusively show that the inhibitors were decreasing the activity of Rubisco

(Parry *et al.*, 2002). The reduced turgor pressure that often results from water

limitation can cause changes in chloroplastic pH and ion concentrations due to the

increased permeability of chloroplastic membranes. It is thought that these

changes can contribute to RuBisCo inactivation (Nilsen and Orcutt, 1996). There

are also still some questions about the effects of water stress on cellular RuBP.

The mechanisms of both decreased RuBisCo activity and RuBP regeneration

under drought stress are not well understood (Flexas *et al.*, 2004); (Flexas and

Medrano, 2002; Bota *et al.*, 2004; Flexas *et al.*, 2004). The reduced enzyme

functionality coupled with multiple feedback mechanisms such as reduced

carbohydrate availability or poor generation of ATP will all play a role in

effecting carbon metabolism rates, under drought stress (Chaves et al., 2003).

Debate on whether the primary limiting factors to carbon metabolism rates are

stomatal or non-stomatal to photosynthesis under drought stress still exists.

Recent evidence indicating an increase in carbon availability under drought stress

due to processes such as growth reduction and osmotic adjustment coupled with

starch degradation may offset stomatal or non-stomatal limitations to

photosynthesis (Hummel et al., 2010). Regardless, creeping bentgrass plants that

are able to use multiple mechanisms to maintain the stability of these processes

under drought stress conditions are considered to be more drought tolerant.

Osmolyte Accumulation and Osmotic Adjustment (OA)

Carbohydrate metabolism will not only determine growth rates and energy production as discussed above but will also be a factor in determining the availability of carbon skeletons for other stress protective mechanisms. Carbohydrate availability is a main factor in determining a plant's ability to adequately actively or inactively accumulate free osmolytes in the process known as osmotic adjustment (OA). OA is a drought tolerance mechanism in which plants accumulate small molecular weight metabolites such as sugars, organic acids, and amino acids to decrease the osmotic potential of the cell for water retention and maintenance of turgor pressure (Turner and Jones, 1980). The extent of OA is highly dependent on factors such as environmental conditions, stress duration, stress severity, plant organ, and genetic variation within plant species and cultivars (Morgan, 1984). Therefore, OA is a drought tolerance mechanism that is widely used by multiple grass species, including creeping bentgrass, to overcome stress in multiple environments. Genetic variation does exist within creeping bentgrass types (DaCosta and Huang, 2006) and therefore is a valuable parameter to measure for physiological evaluation of the sensitivity of a grass to drought stress and to select for drought tolerant germplasm.

Osmolytes important in OA in many plant species are generally categorized as protein and non-protein amino acids, other amine containing compounds and derivatives, soluble carbohydrates, organic acids and alcohols, and ions (Zhang et al., 1999). Primary examples of important osmolytes contributing to OA in creeping bentgrass are proline, glycine betaine, and sucrose

(DaCosta and Huang, 2006). Selecting for varieties with increased accumulation of these osmolytes through breeding or genetic manipulation has been a successful way to improve plant tolerance of drought stress. For instance, transgenically engineered maize plants (*Zea maize* L.) containing a gene to enhance GB synthesis and accumulation exhibited greater drought tolerance and had higher grain yield under drought stress than wild-type plants (Quan et al., 2004). Similarly, proline accumulation has also been shown to effectively confer drought tolerance in several transgenic lines of different species. The D1-pyrroline-5-carboxylate synthetase genes, AtP5CS from *A. thaliana* and OsP5CS from *O. sativa*, were both effective in improving drought tolerance in petunia (*Petunia x hybrida*) (Yamada *et al.*, 2005). Soybean (*Glycine max*) plants were transformed with the cDNA for L-1-pyrroline-5-carboxylate reductase (P5CR), an enzyme involved in proline biosynthesis, in the sense and antisense directions. Sense transformants exposed to drought exhibited the least water loss, greatest proline levels, and had higher levels of NADP+ to act as electron acceptors for PSII and enhanced photosynthesis compared to the antisense plants (De Ronde *et al.*, 2004; Simon-Sarkadi *et al.*, 2005). In addition, to differences in osmolyte accumulation, plants able to maintain the functionality and content of various membrane transporters such as ABC transporters and aquaporins may play a role in the extent and speed of OA (Conde et al., 2011).

Cellular Membrane Stability

Drought tolerance mechanisms such as OA would not be effective if the damage to plant cell membranes did not allow for maintenance of cellular turgor

pressure or compartmentalization of cell constiuents. During drought stress,

membrane damage can become a severely limiting factor for cellular health.

Membrane composition and properties are highly sensitive to dehydration and a

decrease in membrane stability is associated with loss of electrolytes and leakage

of other celluar constituents. In response to moderate drought, the lipid content of

membranes has been shown to decline due to the inhibition of lipid biosynthetic

pathways as well as stimulation of lipolytic and peroxidative activities (Fu and

Huang, 2001). In response to cellular drying, the total lipid content may decline

and there is a significant change in lipid composition. Membrane polarity also is

affected by drought stress, under which a decrease in polar lipids can be observed

(Yordanov *et al.*, 2000). The compositional changes include an increase in

desaturated fatty acids and an altered balance between lipid types such as

monogalactosyl-diacylglycerol (MGDG) and digalactosyl-diacylglycerol

(DGDG). In grasses, drought stress reduces the ratio of MGDG to DGDG. This

ratio is important in determining the structure of lipid bilayers, since MGDG

tends to form hexagonal phase structures and DGDG forms lamellar phases. Thus,

the alteration of this ratio causes reduced cellular membrane stability and

inhibition of proper functioning of photosynthetic membranes (Gigon et al.,

2004). Membrane composition and stability will also have a direct impact on the

functioning of major processes that are primarily membrane bound such as the

transport of electrons in energy generating processes by membrane proteins in

electron transport chains (Yardanov et al., 2000) as well as cellular water and

nutrient transport by transporters such as aquaporins (Maurel, 2007).

In addition to altered composition and permeability, recent studies have implicated membranes to be signaling indicators of drought stress. In addition to secondary messengers such as Ca^{2+} and cAMP, lipids such as phosphatidic acid (PA) have been recognized as signaling molecules. PA is formed by the cleavage by phospholipase D (PLDα) of structural lipids, such as those in membranes, to form PA and free polar head groups. The presence of free PA is a rapid and transient signal that triggers protein kinases and other cellular response mechanisms. Flux through the PLD pathway triggers an ABA response and the production of PLD is induced by ABA (Testerink and Munnik, 2005). Thus, maintenance of cellular membrane stability by grasses is a drought tolerance mechanism that has a broad range of implications in imparting stress tolerance. Identification of grasses that have enhanced membrane stability under drought stress can be determined by measurement of leaf or root electrolyte leakage (Blum and Ebercon, 1981).

Reactive Oxygen Species (ROS) Scavenging

During drought stress, ROS accumulate due to a number of reasons including anabolic processes, byproducts of respiration, cessation of adequate flux through photochemical pathways, and they may be actively produced by plants for signaling purposes (Mittler, 2002). Regardless of how they are produced, the hyperaccumulation of ROS under drought conditions can become toxic to plant cells. Thus, plants contain a wide range of ROS scavenging systems to prevent damage caused by ROS buildup. Plants that have the most effective or multiple pathways within their antioxidant system are typically more drought tolerant,

since the removal of ROS is necessary to prevent oxidative damage caused by their accumulation (Apel and Hirt, 2004). The accumulation of ROS caused by heat and drought stress are alleviated mainly by the induction of gene expression coding for antioxidant enzymes such as superoxide dismutases (SOD), catalases, glutathione-S-transferases (GST), ascorbate peroxidases (APX), and glutathione peroxidases that break down and remove ROS (Ramanjulu and Bartels, 2002; Sharma and Dubey, 2005).

Among other antioxidant mechanisms, H_2O_2 detoxication by different APX isoforms plays an important role in drought tolerance. Water deficit induced increases in transcript accumulation of APX genes in cowpea (*Vigna unguiculata*) cultivars were positively correlated to drought tolerance. Chloroplastic APX genes responded early to progressive water deficit, suggesting that the enzymes detoxify ROS at their production site (D'Arcy-Lameta *et al.*, 2006). Superoxide dismutase (SOD) enzymes also are highly upregulated during drought stress and have been shown to successfully reduce oxidative damage. Under the control of an oxidative stress-inducible promoter, rice plants (*Oryza sativa* L.) expressing a pea manganese superoxide dismutase (MnSOD) in chloroplasts were shown to have less electrolyte leakage and higher photosynthesis rates than wild type plants (Wang *et al.*, 2005). Catalase (CAT) is also an antioxidant enzyme, which functions to remove H_2O_2. The expression of CAT genes in wheat was found to be complexly regulated by drought stress. CAT is an enzyme that is sensitive to degradation due to drought stress has been observed in several plant species (Luna et al 2004). Plants that are able to maintain or have greater antioxidant enzyme

functions of those that are sensitive to drought stress, such as CAT, may be more tolerance of drought stress than their counterparts that may lose antioxidant enzyme activity and content.

In addition, many non-enzymatic gene products have been shown to be involved in ROS scavenging either directly by actively scavenging or indirectly by inducing gene expression of other antioxidants. For instance, calcium, ABA, ethylene, and salicylic acid were all shown to protect plants from heat and drought stress-induced oxidative damage (Larkindale and Knight, 2002), as well as nitric oxide (Hung and Kao, 2004). Other non-enzymatic ROS scavenging metabolites are isoprene (Penuelas *et al.*, 2005), α-tocopherol (Munne-Bosch, 2005), ascorbate (AA), reduced glutathione (GSH), and pigments such as carotenoids and flavonols (Jiang and Zhang, 2002). There are also various compounds that induce the expression of antioxidant enzymes such as proline, which accumulates under drought stress conditions (Kocsy *et al.*, 2005).

Other stress protective proteins and metabolites

Stress protective proteins such as dehydrins, chaperones, protease inhibitors, and others serve to assist protein folding, prevent denaturation of individual protein subunits, protein complexes (e.g. membrane bound or cytoplasmic), and other cellular structures in order to maintain their functionality (Close, 1996; Ingram and Bartels, 1996). Chaperones may have specific targets and have a large effect on the health of major metabolic pathways. For instance, in drought tolerant compared to sensitive wheat cultivars, maintenance of CLP

proteases, some heat shock proteins, RuBisCo activase, RuBisCo binding proteins, and dehydrins were important in determining drought tolerance (Demirevska, et al., 2008). Therefore, maintenance of these proteins may play a role in maintinaing photosynthesis rates in wheat exposed to drought stress. The function of other proteins up-regulated by drought stress such as universal stress proteins are thought to be involved in stress protection, but the role in plants is relatively unknown (Isokpehi, et al., 2011).

If protective mechanisms are overwhelmed and proteins do become damaged by drought stress, quick protein turnover is considered to be a drought tolerance mechanism. A rapid stimulation of protease activity for removal of irreversibly damaged and unfunctional proteins may confer drought tolerance. This allows for rapid replacement with newly synthesized, functional proteins. In addition to replacement, quick protein turnover is considered to by a valuable recycling process. Plants may utilize by-products of protein degradtation for other biochemical pathways important in the defense response. For instance, amino acids and amine side chains may be used in osmotic adjustment or to synthesis nitrogenous secondary metabolites (Hieng et al., 2004). Plants possess a complex network of pathways enabling them to degrade damaged proteins, including pathways that are drought specific or are shared among responses to other abiotic stresses (Khanna-Chopra et al., 1999). Plants stimulate degradation by up-regulating genes and proteins involved in ubiquitin activation tagging, compilation of proteasomes, and change in the content and activity of various ligases, phosphatases, proteases, and other enzymes (Chaves et al., 2003).

Genomic Localization of Important Drought Tolerance Genes

A comprehensive approach to evaluating complex drought tolerance traits that aims to understand all aspects of a drought tolerance mechanism from a whole-plant level to biochemical responses is essential for utilization of this knowledge in an applied agricultural setting. Moving the conceptual knowledge into the industrial forefront is largely achieved by plant breeding efforts. Breeding efficiency can be significantly amplified by utilizing practices that involve knowledge of genetic regions controlling desirable traits. In creeping bentgrass, the whole genome has not yet been sequenced. Therefore, the main method available in this species to identify important genomic regions is by evaluating plants for quantitative trait loci (QTL) based on chromosomal linkage maps.

The identification of QTL is a method of associating a large genomic region of a species with the control and inheritance of a quantitative, phenotypically measurable trait. Quantitative traits are those that are controlled by many genes and biochemical processes such as traits affecting growth, yield, and stress resistance. Once identified, QTLs of desirable traits can be later evaluated for use in marker assisted selection, specific genes underlying the QTLs, or for analysis of genomic synteny with related plant species. Therefore, the ultimate goal of QTL identification is for future use as a tool for genomic selection within molecular breeding (Edwards et al., 1987). Opponents to QTL mapping have argued that the method may not be worthwhile due to the laborious nature of genotyping and phenotyping, the marginal level of progress in fine mapping for

gene identification, and the potential for false QTLs (Kearsey and Farquhar, 1998; Borevitz and Chory, 2004; Tuberosa and Salvi, 2006). However to date, advances in genomics such as high throughput technologies, greater availability of data for detection of synteny, and several recent successes in fine mapping have allowed QTL detection and genomic selection utilizing markers to be considered the future of plant breeding (Ren et al., 2010; Yang et al., 2010; Lorenz et al., 2011; Salunkhe et al., 2011).

QTLs have been successfully identified for a wide range of important agronomic traits such as those responsible for yield and stress resistances (Vinocur and Altman, 2005) and linking genomics to physiological traits is of immense importance for production of stress tolerant crops (Edmeades et al., 2004). Drought stress is the foremost abiotic stress that limits the growth and productivity of many plant species. Identification of QTLs for various drought tolerance traits has been achieved in several major crop species such as corn (*Zea mays* L., Ribaut et al., 1997; Hao et al., 2010), wheat (*Triticum aestivum* L., Dashti et al., 2007), and rice (*Oryza sativa* L., Price et al., 2002), as well as model species such as Arabidopsis (*Arabidopsis thaliana* L., Juenger et al., 2005). In comparison to annual crops, relatively little information is available regarding genomic information or QTLs for drought tolerance traits in grass species, particularly those used as turfgrass. In turfgrasses, QTLs have mainly been identified for prevalent biotic diseases such as dollar spot in creeping bentgrass (Bonos, 2006; Chakraborty et al., 2006) and for gray leaf spot (Jo and Jung, 2006; Curley et al., 2008) and crown rust resistance in perennial ryegrass (*Lolium*

perenne) (Sim et al., 2007). Other valuable morphological characteristics such as seed yield (Brown et al., 2010) are being evaluated for QTL identification. Jo and Jung (2006) identified four QTLs for gray leaf spot resistance. Those QTL markers for disease resistance have a great potential to be utilized in breeding improvement for disease resistance. This approach may also be effective for developing markers linked to abiotic stress tolerance in turfgrass. Knowledge of QTLs for abiotic stress tolerance traits in turfgrasses is severely lacking and the turfgrass industry would benefit greatly from QTLs for drought tolerance in creeping bentgrass.

Objectives

The main goals of this thesis were to evaluate how creeping bentgrass drought tolerance can be improved by genetic modification and genomic localization of drought tolerance traits. Part I will evaluate how increasing the endogenous level of a group of plant hormones, the cytokinins, will affect the drought tolerance response of creeping bentgrass. Drought injury symptoms have been associated with an inhibition in cytokinin synthesis and maintenance of endogenous cytokinin is associated with alleviation of drought damage.Therefore, the benefits of maintenance of cytokinins was determined by a comprehensive evaluation of transgenic creeping bentgrass containing a gene promoting cytokinin biosynthesis during drought stress on 1) whole-plant physiology 2) proteomic 3) metabolic and 4) genetic responses. Part II will evaluate major

physiological responses to drought stress in a bentgrass population in order to

identify chromosomal regions associated with drought tolerance traits.

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PART I - DROUGHT STRESS RESPONSES OF CREEPING
BENTGRASS CONTAINING AN IPT TRANSGENE PROMOTING
CYTOKININ BIOSYNTHESIS

CHAPTER 1

**EFFECTS OF SAG12-IPT AND HSP18.2-IPT EXPRESSION ON
CYTOKININ PRODUCTION, ROOT GROWTH AND LEAF
SENESCENCE IN CREEPING BENTGRASS EXPOSED TO DROUGHT
STRESS**

INTRODUCTION

Drought is a detrimental abiotic stress for plant growth, including perennial turfgrass species. A typical drought stress symptom in turfgrass is a decline in turf quality (TQ) resulting from leaf senescence, slow shoot and root growth, and leaf desiccation (Fry and Huang, 2004). Plant adaptation to drought stress has been associated with the hormonal regulation of these processes. Changes in the level and proportion of endogenous phytohormones, such as cytokinins (CK) and abscisic acid (ABA), affect some stress adaptation mechanisms, including stomatal closure, alteration of root:shoot ratios, carbon partitioning, and the degree of leaf senescence and root mortality (Davies et al., 1994). CK are a major class of plant hormones that regulate or effect cellular functions during plant growth and development, including cell division, leaf senescence, and tiller and root growth and production (Mok and Mok, 1994, 2001). Since it was found that ABA was highly regulated and response to drought stress, most studies analyzing phytohormone responses to drought stress have focused on ABA and its involvement in regulating stomatal closure (Bray, 1993; Chaves et al., 2003; Kramer and Boyer, 1995; Marrion-Poll and Leung, 2006). Some studies in annual crops have implicated CK in the coordination of plant responses to environmental stresses, including drought stress (Chaves et al., 2003). How CK may regulate drought tolerance, particularly in perennial grasses, is not well understood.

To study the effects of CK metabolism on stress tolerance and the mechanisms of CK regulation of stress tolerance, two approaches have been employed: exogenous application of CK and transgenic modification of endogenous CK levels. Generally, plants maintaining or exposed to higher levels of CK, either by alterations of endogenous production by transgenic methods or by exogenous application, exhibit improved tolerance to different stresses. For example, creeping bentgrass plants that were treated with a CK injection into the root zones showed increases in TQ and photochemical efficiency (Fv/Fm) largely due to the alleviation of heat-induced root mortality and increased antioxidant activity (Liu et al., 2002; Liu and Huang, 2002). Likewise, Zhang and Ervin (2004) demonstrated that creeping bentgrass showed improved TQ under drought stress when treated with an exogenous application of a seaweed extract containing CK. However, the exogenous application of hormones does not always provide the same physiological effects as changes in endogenous levels of hormones (Okamoto et al., 2010). Thus, internal modifications of CK levels may be more useful for understanding how CK regulates drought tolerance. The CK gene used in this study encodes adenine isopentenyl transferase (ipt), which catalyzes the formation of isopentenyladenosine-5′-monophosphate from 5′AMP and isopentenylpyrophosphate, a key enzyme involved in the rate-limiting step leading to de novo CK biosynthesis (Medford et al., 1989; Morris, 1995). Transgenic plants expressing the ipt gene exhibit increased tolerance to different stresses in some plant species, including drought in petunia (Petunia ×hybrida) (Dervinis, 1999), lettuce (Lactuca sativa) (McCabe et al., 2001), and tobacco

(Nicotiana tabacum) (Rivero et al., 2007), flooding in arabidopsis (Arabidopsis thaliana) (Huynh et al., 2005; Zhang et al., 2000), cold in tall fescue (Festuca arundinacea) (Hu et al., 2005), and nutrient deficiency in tobacco (Jordi et al., 2000). In ipt transgenic lettuce, the observed increases in drought tolerance of the transgenic plants were attributed to hexose accumulation (McCabe et al., 2001). Rivero et al. (2007, 2009) reported that ipt transgenic tobacco exhibited improved drought tolerance due to delayed leaf senescence, changes in photorespiration, protection of photosynthesis, and increased water use efficiency. Havlova et al. (2008) transformed tobacco with a gene encoding transzeatin O-glucosyltransferase (ZOG1) to increase endogenous CK O-glucosides, a storage form of CK, and found delayed leaf senescence of older leaves, decreases in cytokinin oxidase activity during drought stress, and improvement in postdrought recovery compared with wild-type controls.

The benefits of elevated CK levels under drought stress in a perennial grass species maintained under turf conditions where leaf senescence is a primary concern for TQ have not yet been evaluated and may be different from annual crops such as tobacco and lettuce. In addition, the senescence of older leaves is known to be a drought survival mechanism similar to dormancy in many crop species. This mechanism may be desirable in some plants as a way to redirect energy reserves to younger leaves or toward plant reproduction, thus increasing yield or for survival at the whole plant level. However, it has also been shown that maintenance of older leaves by avoidance of senescence is beneficial for additional energy produced by a greater amount of photosynthetic source leaves

(Rivero et al., 2007). Furthermore, due to the cessation of significant growth relative to younger leaves, older leaves do not typically act as much of a sink to draw nutrients away from a plant, reducing energy that could have gone toward drought tolerance mechanisms (Khan, 1981). In addition, perennial turfgrass species performance is not based on yield but on aesthetic appearance for which leaf senescence is undesirable. Limited information is available about the root growth characteristics of ipt plants, which is an important factor influencing water uptake under drought stress. With an aim to examine the effects of CK effects on drought performance in perennial turfgrass species, we transformed a widely used cool-season turfgrass species, creeping bentgrass, using the *ipt* gene ligated to a senescence-associated promoter, *SAG12* (Gan and Amasino, 1995) and a heat shock promoter, HSP18.2 (Takahashi and Komeda, 1989). The senescence- and stress-inducible promoters circumvent the abnormal growth problems associated with the overproduction of CK in transgenic plants containing the ipt gene driven by constitutive promoters (Dansanko et al., 2003; Gan and Amasino, 1995; Schnablova et al., 2006; Yoshida and Shinmyo, 2000). In previous studies, *SAG12-ipt* transgenic creeping bentgrass exhibited improved growth under heat stress (Xu et al., 2009) and nutrient deficiency (Zhang et al., 2010) in association with increased tiller production, root growth, and root:shoot ratio. The objectives of this study were to investigate whether expression of the *ipt* gene-promoting CK synthesis driven by senescence- and/or stress-inducible promoters would improve drought performance in creeping bentgrass, and to examine shoot and root growth

responses to drought stress associated with changes in endogenous production of

CK and the ratio of CK and ABA due to the *ipt* transformation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Transgenic plants were developed by the Agrobacterium (*Agrobacterium tumefaciens*) transformation method as described in Xing et al. 2010 and Xu et al. 2009. Plant materials included *SAG12-ipt* transgenic lines (S7, S8, S16, S25, S32, S37, S40, S41, S43, S55, S97, and S99), *HSP18.2-ipt* transgenic lines (H13, H27, H29, H31, H37, H42, and H43), the wild-type cultivar 'Penncross' (WT), and a null transformant (NT) control line of 'Penncross' that was transformed with an empty plasmid vector without the *ipt* gene. The transgenic plant lines used in this study were verified, by northern analysis, to be transformed and to contain the *ipt* gene, whereas the WT and NT plant lines did not contain the transgene, as shown in Xu et al. 2009. In addition, all material has been clonally propagated since northern confirmation analysis to negate any possibility of transgene loss due to sexual reproduction or recombination. Plant materials were established in eight large plastic containers (54 cm long, 42 cm wide, and 14 cm tall) filled with fine sand (0.125 mm particle size) with ≈10 individual plants from the control and each transgenic line. Plants were grown in a controlled environment growth chamber (GC15; Environmental Growth Chambers, Chagrin Falls, OH) and were allowed to establish for 4 weeks before watering treatment imposition. The growth chambers were set to regulate chamber conditions at a 12-h photoperiod, 50% relative humidity, 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux (*PPF*), and a day/night temperature of 23/20 °C. Plants were watered well and were fertilized with a controlled-release fertilizer (19N–2.6P–10K; Scotts, Marysville, OH) once

during plant establishment in the greenhouse and once before water treatment in the growth chamber. Plants were maintained at ≈3 cm height by hand clipping weekly during the establishment period, but were not trimmed during drought stress treatment.

Watering Treatments

Drought stress was imposed by completely withholding irrigation from four containers for 14 d. The well-watered control plants within four containers received water daily until drainage was observed from each container. Each treatment was replicated four times in four plastic containers. Each container contained plants from each transgenic line, the WT, and the NT control line so that all plant materials were exposed to the same level of soil water availability during drought stress.

Measurements

Soil volumetric water content was determined with the time domain reflectometry method (Topp, 1980) (Trase; Soil Moisture Equipment, Santa Barbara, CA). Two-pronged waveguide probes 20 cm in length were buried horizontally in the middle of the root-zone media in each container and measurements were taken periodically during the 14-d treatment period.

Overall turf performance was evaluated by visually rating turf quality (TQ). TQ was visually rated every 2 d based on turf uniformity, color, and density 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 dense turf (Turgeon, 2008). Total root

length and biomass and total shoot biomass were determined at the end of drought stress (14 d) by destructive sampling. Roots were washed free of sand and separated from shoots at the crown. Total root length was calculated by separating the fresh roots on a flatbed scanner (4490; Epson, Long Beach, CA) and the total length was calculated with WinRhizo software (Regent Instruments, Loretteville, Canada). Subsequently, all plant biomass was dried in an 80 °C oven for 72 h for dry weight (DW) determination. Root-to-shoot ratio was calculated as the ratio of root DW to shoot DW that included all tissues of the whole plant.

Relative water content (RWC) of leaves was measured as an indicator of leaf hydration status. Leaf RWC was calculated based on fresh (FW), turgid (TW), and DW of ≈0.1 g of leaf samples using the following formula: (FW − DW)/(TW − DW) × 100. Leaf FW was determined on a mass balance immediately after being excised from the plants. Turgid weights were determined after soaking the leaves in deionized water for 12 h in a closed petri dish at 4 °C and weighing them immediately after being blotted dry. Leaves were then dried in an 80 °C oven for at least 72 h before being weighed for DW (Barrs and Weatherley, 1962).

Leaf Chl content and Fv/Fm were measured to evaluate leaf senescence. A hand-held leaf Chl meter (SPAD-502; Spectrum Technologies, Plainfield, IL) was used to measure Chl content on two subsamples taken per plant. The Chl meter gives an index of total leaf Chl content. The index values were converted to Chl content and were expressed as milligrams per gram DW using a standard curve

constructed with actual Chl content against the index values. Chlorophyll of leaves for the standard curve was extracted in dimethyl sulfoxide, and the absorbance was measured at 663 and 645 nm with a spectrophotometer (Genesys 2; Spectronic Instruments, Rochester, NY). The content of Chl was calculated using the formula described in Arnon (1949). Photochemical efficiency was evaluated as a ratio of the variable fluorescence (Fv) to the maximal fluorescence (Fm) value determined using a Chl fluorescence meter (Fim 1500; Dynamax, Houston). Leaf clips were used to adapt individual leaves to darkness for 30 min before reading the Fv/Fm ratio with the fluorescence meter. Two subsamples were taken per plant at each sampling day.

Cytokinin and ABA content was measured to evaluate changes in endogenous content and the ratio of these hormones. Hormone extraction and quantification was determined by an indirect enzyme-linked immunoabsorbent assay method described in Setter et al. (2001) with modifications (Wang et al., 2003). Samples were extracted in 80% (v/v) methanol and purified with reverse-phase C18 columns. Hydrophilic contaminants were removed with a solution of 20% methanol and 80% aqueous triethylamine (10 mm TEA, pH 3.5). Subsequently, the CK fraction was eluted with 30% methanol and 70% TEA, and ABA fractions were eluted with 55% methanol.

Experimental Design and Statistical Analysis

The experimental design was a split-plot design with irrigation treatments as the main plots and plant materials as the subplots, with four replicates for each

irrigation treatment and grass material. Effects of watering treatment, plant materials, and corresponding interactions were determined by analysis of variance (ANOVA) according to the general linear model procedure of SAS (version 9.0; SAS Institute, Cary, NC). Differences between watering treatments and plant means were separated by Fisher's protected least significance difference (lsd) test at the 0.05 P level.

RESULTS

Soil Water Status

Soil water content for well-watered plants was maintained at ≈25%. In the drought treatment, soil water content declined to ≈5% by 14 d of drought. Each replication of *SAG12-ipt, HSP18.2-ipt* transgenic lines, NT, and WT plants were exposed to the same level of drought stress because they were planted in the same container, which allowed for an examination of drought responses of different plant materials to the same level of water deficit (Fig. 1).

Relative Water Content (RWC)

Well-watered plants maintained RWC levels at 85% to 90% throughout the duration of the experiment, with no significant difference between plant lines (data not shown). The average RWC of all well-watered plant lines as sampled on 12 d of water treatment (87%) is presented as a threshold value in Fig. 2. RWC declined in response to drought stress in all plant lines. Significant differences in RWC between lines were not observed until 12 d of drought stress when five *SAG12-ipt* lines (S16, S37, S40, S55, and S8) and one *HSP18.2-ipt* line (H31) had higher RWC values than NT and WT plants (Fig. 2). Most of the transgenic-*ipt* lines maintained RWC at or above 70%, while the RWC of NT and WT plants were below this level at 12 d of drought.

Turf Quality (TQ)

Well-watered plants generally did not exhibit significant differences in TQ among transgenic-*ipt*, WT, and NT throughout the experimental period, except at 6 and 14 d of treatment due to the lower TQ of H43 (Fig. 3). Most *SAG12-ipt* lines had significantly higher TQ from 8 through 14 d of drought relative to WT and NT plants, except for lines S25 and S37 (Fig. 3). Plant lines exposed to drought stress for 14 d also exhibited significant variation in the degree of decline in TQ. Turf quality ratings for WT and NT plants dropped to below the minimal acceptable level of 6.0 at 8 d of treatment, whereas most of the *SAG12-ipt* lines did not fall to below this level until 14 d of drought. Most *HSP18.2-ipt* lines started to fall below the acceptable level after 10 d of drought. Differences in TQ of *HSP18.2-ipt* lines relative to NT and WT were less pronounced; however, H31 and H29 had significantly higher TQ ratings than the NT control at 14 d of drought.

Total Chl Content

Leaf Chl content did not vary between plant lines and remained constant under well-watered conditions (Fig. 4, A and B). Among plant lines exposed to drought stress, significant differences occurred after 2, 6, and 12 d of drought (Fig. 4, A and B). Leaf Chl content declined in all plant lines in response to drought stress, but the declines in *SAG12-ipt* lines were less pronounced than for NT and WT plants. The Chl content of NT and WT plants declined by an average of 68% at 12 d of drought, whereas the Chl content of *SAG-ipt* lines declined by

an average of 50%. Transgenic lines H27, S39, S25, and S41 had the greatest

amount of Chl, and NT plants had the lowest Chl content under drought stress.

Photochemical Efficiency (F_v/F_m)

Under well-watered conditions, no significant differences in Fv/Fm were

detected between the plant lines, which maintained an average of ≈0.80

throughout the duration of the experimental period (Fig. 5, A and B). Drought

stress caused a significant decline in Fv/Fm in all plant lines (Fig. 5B). *HSP18.2-*

ipt and *SAG12-ipt* lines exhibited variation in Fv/Fm, and several *ipt* lines

maintained significantly higher Fv/Fm levels compared with NT and WT plants at

6, 9, and 14 d of drought. By 14 d of drought stress, all transgenic lines had

significantly higher Fv/Fm than the NT line.

Root Growth and Root:Shoot Ratio

Plant lines H13, H29, H31, S16, S25, S32, S43, S55, S7, S97, and S99 had

significantly higher total root biomass than the WT under drought stress. The

same lines, with the addition of line S37 and the exception of lines H29, H31, S7,

and S97, exhibited greater total root length, which can most likely be attributed to

the additional root biomass. The root:shoot ratio was analyzed to normalize

differences between transgenic-*ipt* and control lines under optimal conditions

such as due to differential tiller numbers (Xu et al., 2009). Root:shoot ratios were

generally higher in transgenic-*ipt* lines compared with NT and WT after 14 d of

drought (Fig. 6C); for example, lines S25 and S7 had root:shoot ratios of ≈0.25,

whereas the average root:shoot ratio of NT and WT was ≈0.075.The root:shoot ratio averaged ≈0.48 for the *SAG12-ipt*, NT, and WT under well-watered conditions (Fig. 6). At 14 d of drought stress, significant differences in root:shoot ratio were observed between plant lines (Fig. 6). Transgenic-*ipt* lines H29, H31, S16, S25, S32, S43, S7, S97, and S99 had significantly higher root:shoot ratios compared with the NT and WT plants. The highest root:shoot ratio in drought-stressed plants was found in transgenic line S25 and S7, at ≈0.25, whereas the lowest ratio was in NT plants, at ≈0.05.

Leaf iPA and ABA Content

Leaf iPA content of well-watered plants did not differ significantly between plant lines, which averaged ≈30 pmol·g^{-1} DW in leaves (Fig. 7A, threshold). After 14 d of drought, iPA content declined in all plants. Seven of the 12 *SAG12-ipt* lines (S25, S37, S40, S41, S43, S7, and S99) had a significantly higher leaf iPA content compared with NT and WT, although variation in iPA accumulation existed among these transgenic-*ipt* lines (Fig. 7A). The *SAG12-ipt* lines that were significantly different from NT and WT had an average iPA content more than four times higher, at 9.0 pmol·g^{-1} DW in transgenic-*ipt* lines compared with 2.2 pmol·g^{-1} DW in WT and NT plants. Slight increases in iPA content were found between the *HSP18.2-ipt* line and the control lines; however, these were not statistically different. Under well-watered conditions, leaf ABA content was ≈25 pmol·g^{-1} DW (Fig. 7B). Drought stress resulted in an accumulation in leaf ABA content above this control level. Transgenic-*ipt* lines

H13, H29, H42, S25, S32, S40, S41, S55, S7, S97, and S99 maintained leaf ABA levels significantly lower than NT and WT plants at 14 d of drought stress (Fig. 7B).

Root iPA and ABA Content

Root iPA content did not significantly differ between the NT, WT, and transgenic lines under well-watered conditions, which averaged 40 pmol·g^{-1} DW (Fig. 8A, threshold). At 14 d of drought stress, root iPA content decreased significantly in WT, NT, and most of the *SAG12-ipt* plants, but was maintained at the well-watered level in S40, S55, and S8. Root iPA content in 11 of 19 transgenic lines (H27, H31, H39, H43, S25, S37, S40, S41, S43, S7, S99, S55, S8, and S97), was statistically higher than in the NT and WT lines, and averaged four times the NT and WT levels. The total additive iPA content in leaves and roots was significantly higher in most transgenic-*ipt* plants than in NT and WT plants under drought stress. Root ABA content did not accumulate due to drought stress relative to the control level of 40 pmol·g^{-1} DW (Fig. 8B). Transgenic lines H13, H29, H31, S25, S37, S43, and S97 had significantly lower ABA than the NT and WT plants, whereas H43 had significantly higher root ABA (Fig. 8B).

DISCUSSION

Several *ipt*-transgenic lines exhibited improvement in drought performance as indicated by significantly greater TQ, Fv/Fm, Chl content, and RWC under drought stress. Overall, ipt expression in creeping bentgrass was effective in promoting better turf performance and alleviating drought-induced physiological changes such as leaf senescence, although significant variation was observed among the *ipt* lines and between the different promoters. The variation in turf performance between transgenic-ipt lines of the same promoter could be due to differential genomic insertion locations of the transgene (Bettany et al., 1998) or due to somaclonal variation (Larkin and Scowcroft, 1981), which may cause differences in transgene expression patterns. Greater differences in TQ, Chl content, Fv/Fm, and RWC were observed in the *SAG12-ipt* plants than in the *HSP18.2-ipt* plants, relative to the control lines. This could be due to lower expression of the *HSP18.2* promoter, leading to a smaller increase in iPA content in roots and shoots compared with the SAG12-ipt lines. For example, Sakuma et al. (2006) found that the *HSP18.2* was not highly expressed under drought stress compared with heat shock treatment. However, in our study, because root iPA levels were significantly higher in HSP18.2-ipt lines, other secondary cellular stresses could have activated the *HSP18.2* promoter. Oxidative stress could have contributed to the induction of expression because *HSP18.2* can be activated by hydrogen peroxide (Kovtun et al., 2000). More research is needed to confirm this possibility for *HSP18.2- ipt* lines.

The most pronounced effects of ipt transformation in creeping bentgrass were the increases in total root biomass, root length, and root:shoot ratio. The improvement in rooting characteristics may enhance water uptake, and thus, the ipt transgenic plants, with a more extensive root system, may be more effective in obtaining water from drying soils and delaying physiological changes from drought stress such as leaf senescence and crown dormancy. Nevertheless, previous studies reported decreased root production with increased endogenous CK in dicot species such as tobacco and arabidopsis (Clark et al., 2004; Luo et al., 2005; Medford et al., 1989), and several studies reported reductions in root growth in plants transformed with ipt driven by constitutive promoters (Hewelt et al., 1994; Van Loven et al., 1993). Plants transformed with ipt using constitutive promoters may overproduce CK, which results in root growth inhibition (Gan and Amasino, 1995). Constitutive expression of the ipt gene has been found to elevate endogenous CK levels sufficiently to cause mutation and growth deformation (Klee, 1994). In our study, the ipt transgene was ligated to a stress-inducible promoter for autoregulation of ipt expression that prevents overproduction of CK, and regulates production of CK only after stress is initiated, resulting in limited CK accumulation compared with constitutive expression (Gan and Amasino, 1995; Verdonk et al., 2008). In addition, the difference in the effect of CK on root growth in dicots, and what we observed in our study with a grass species, suggests that CK may regulate root growth differently between plants with tap root systems and those with fibrous root systems (Aloni et al., 2006).

The increases in total root biomass production in our study may be due to increases in root production associated with the stimulation of tiller formation in SAG12-ipt transgenic creeping bentgrass, as reported by Xu et al. (2009). Moreover, Aloni et al. (2006) showed that CK played a significant role in promoting root development, differentiation, and architecture. Specifically, they found that elevated root CK levels in root tips, as controlled by ipt genes, may cause root apical dominance and may allow primary roots to reach water in deeper soil layers. Increased apical dominance promoted primary root growth as opposed to lateral roots. The maintenance of greater root:shoot ratios under drought stress could be at least in part due to enhanced root survival, root production, and/or root elongation due to the expression of ipt in creeping bentgrass under drought stress. Root:shoot ratio has been shown to be an effective selection method in breeding for drought tolerance of perennial turfgrasses such as tall fescue (Karcher et al., 2008). In addition, our results are in agreement with other studies in creeping bentgrass in that an exogenous application of CK (Liu and Huang, 2002) and the presence of SAG12-ipt (Xu et al., 2009) promoted root growth during heat stress conditions.

Endogenous leaf iPA content was lower under heat stress than the values for well-watered plants in creeping bentgrass (Xu et al., 2009), but SAG12-ipt plants still had higher iPA content than NT. Similarly, in this study, total additive iPA content, including that found in leaves and roots, was maintained at higher levels in ipt plants relative to the NT and WT controls under drought stress. Plants that had significantly higher levels of leaf iPA generally had better TQ ratings,

greater Chl content, and higher RWC and Fv/Fm by 14 d of drought than non-transgenic lines and thus had lower levels of drought-induced leaf senescence, although not all transgenic lines that had higher iPA content exhibited improved drought tolerance, as discussed above, or there seemed lack of a direct correlation between iPA content and turf growth when comparing individual transgenic lines. It is possible that other forms of CK such as zeatin riboside and dehydrozeatin riboside may be changed as the result of the transformation, which may account for the variations between transgenic lines. However, the hormone balance of the plant lines may better explain the improvements in physiological attributes under drought stress. This and differences in iPA translocation may be particularly true for the Hsp18.2-ipt lines that had higher root iPA content, but did not accumulate iPA to levels higher than the non-transgenic lines in the leaves. However, considering that iPA is a predominant form of CK in perennial grass species as previously reported (Xu et al., 2009), this study only quantified iPA. Nevertheless, our findings are consistent with previous work done to evaluate exogenous applications of CK, where increased levels of leaf iPA were associated with greater drought tolerance (Zhang and Ervin, 2004). Differences in drought tolerance of bentgrass species have been associated with differences in total CK content in the plant (DaCosta and Huang, 2007). Comparing iPA content in leaves and roots, it seems that at least several transgenic lines such as S32 and S55, S8, and S92 that did not exhibit higher iPA than the NT and WT in leaves had significant increases in roots. Additionally, the poorly performing line H43 most likely had an inadequate hormone balance because it had a relatively high root

ABA content, a low leaf ABA content relative to NT, a high root iPA content, and relatively low leaf iPA content relative to the other lines. The higher amount of ABA in the shoots of H43 relative to the roots may indicate leaf cell damage despite ABA signaling because efficient ABA translocation is required for ABA signaling and an adequate drought tolerance response (Liu and Huang, 2005), and an accumulation of ABA has been shown to occur in less drought-tolerant plants, as discussed below (DaCosta and Huang, 2007). The high total plant iPA of H43 may indicate that the transgene was being expressed at too high of a level. However, further expression analysis studies would be needed to confirm such a conclusion. It is well known that CK are commonly found in the xylem and are thereby transported from the roots, where they are primarily synthesized, to the shoot (Letham and Palni, 1983). Our results suggest that the translocation pattern of iPA between roots and leaves may have been altered in some transgenic plants, which may have caused higher root iPA and may have affected the ABA:CK ratio, resulting in the increases in root growth. However, this cannot be directly concluded because translocation and differences in CK conversion among all forms was not explicitly measured. Alternatively, other mechanisms could be possible because CK have been shown to be involved in other root processes such as promoting vascular differentiation (Aloni et al., 2006), which could have allowed for healthier roots under drought stress and therefore the greater ability of plants to maintain root growth under stress. Alternative to our results, one could argue that CK in the form of iPA is known to cause stomatal opening and reduced root growth, which would reduce drought resistance characteristics. However, it

has been found that the timing of increased CK content, the form of CK present, and the balance of hormones may be more critical in determining stomatal responses during drought stress (Pospisilova et al., 2000, 2005).

Drought stress can lead to an increase in ABA accumulation in various plant species, including creeping bentgrass (DaCosta and Huang, 2007). Most of the transgenic lines had lower ABA content in leaves and roots than the non-transgenic plants. ABA has been associated with the promotion of drought responses, such as stomatal closure, that lead to photosynthesis inhibition (Blackman and Davies, 1983). Lower levels of ABA accumulation have been correlated to drought tolerance in different perennial grass species due to less cellular damage, most likely achieved by alternative drought-adaptive mechanisms (DaCosta and Huang, 2007; Volaire et al., 1998; Wang et al., 2003). Reduced accumulation of ABA may reflect less drought injury in roots and shoots associated with the increases in CK production in transgenic plants. In contrast, some research has reported increased ABA content being associated with greater drought tolerance (Rivero et al., 2007, 2009). Thus, multiple dynamic mechanisms are involved and are not yet fully clear. The higher ABA content in leaves of NT and WT may induce stomatal closure and result in limited photosynthesis during drought stress. The ratio of iPA to ABA was generally higher in leaves and roots of transgenic plants than in the WT and NT plants. Hormone interactions are dynamic because concentrations of other hormones and their proportion between roots and shoots may influence plant growth and development, including leaf senescence (Naqvi, 1995) and stomatal aperture

(FuBeder et al., 1992). In a study with Medicago sativa, plants with a lower ABA content in roots and a higher CK-to-ABA ratio in leaves, as well as higher leaf CK concentrations, maintained photosynthetic activity, leaf conductance, and transpiration flux under drought stress (Goicoechea et al., 2006). The improved drought performance along with the increase in CK-to-ABA ratio in SAG12 and HSP-ipt plants suggests that CK may have an important role in the regulation of drought tolerance in creeping bentgrass through changing the accumulation and the balance with ABA.

In conclusion, transformation of creeping bentgrass with ipt resulted in the improvement in drought performance of creeping bentgrass, as manifested by the higher TQ, Chl content, Fv/Fm, RWC, and root growth compared with the non-transgenic plants. The increases in CK accumulation and the ratio of CK to ABA may be associated with the suppression of leaf senescence and increasing root growth in creeping bentgrass exposed to drought stress. However, further research is required to identify specific mechanisms underlying the effects of ipt expression on drought adaptation in cool-season turfgrasses and other plant species.

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FIGURES

Figure 1. Soil water content (%) in well-watered control and drought treatments in all plant lines of creeping bentgrass. Vertical bars indicate LSD values ($P \leq$ 0.05) for comparison between treatments at a given day of treatment where significant differences were detected.
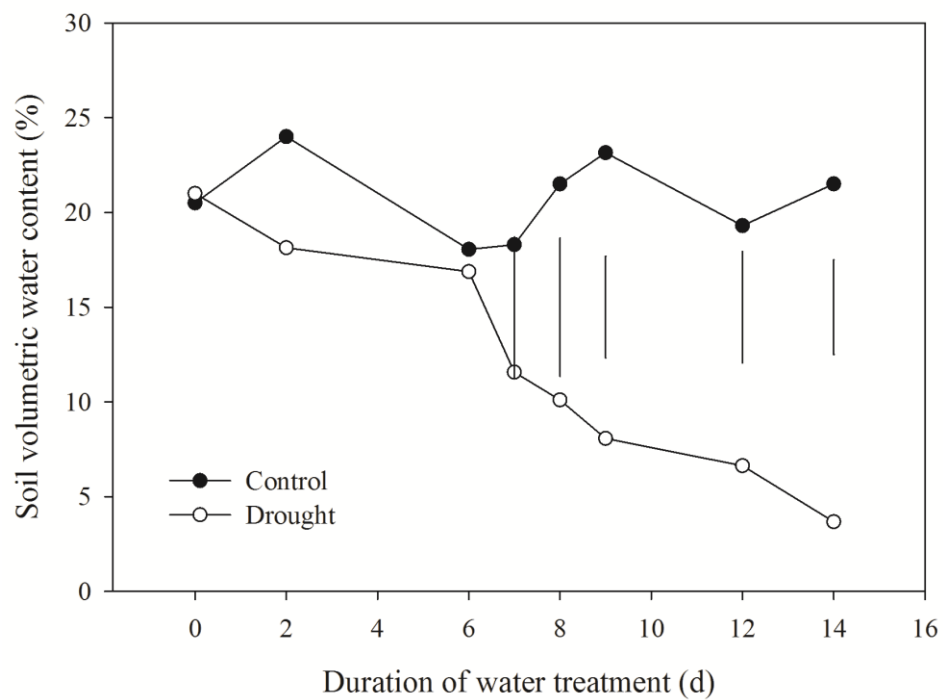
Figure 2. Leaf relative water content (RWC, %) of the null transformant (NT), wild type 'Penncross' (WT), *HSP18.2-ipt* (H lines), and *SAG12-ipt* (S lines) of creeping bentgrass at 12 d of drought stress. The horizontal dashed line represents the average RWC value of all plants under well-watered conditions at 12 d of treatment as transgenic lines, WT, and NT did not differ in RWC in this treatment. The vertical bar indicates LSD value ($P \leq 0.05$) for comparison between plant lines at 12 d of drought stress. Columns marked with an asterisk indicate plant lines exhibiting significant differences from the WT plants.
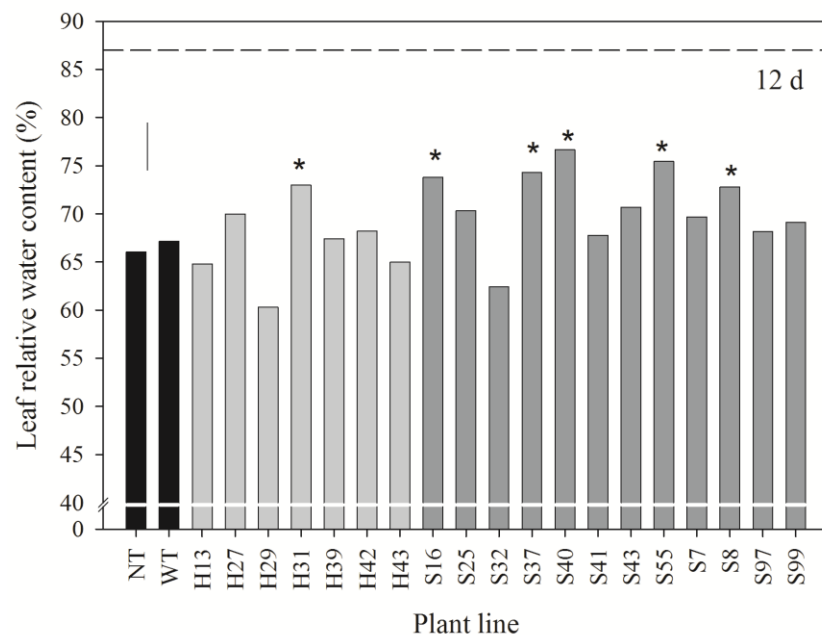
Figure 3. Turf Quality (TQ), a visual rating system on a scale of 1-9, of the null

transformant (NT), wild type 'Penncross' (WT), *HSP18.2-ipt* (H lines), and

SAG12-ipt (S lines) of creeping bentgrass exposed to well-watered conditions (A

and C) and drought stress (B and D). Vertical bars indicate LSD values ($P \leq 0.05$)

for comparison between plants lines at a given day of treatment where significant

differences were detected.

Figure 4. Leaf chlorophyll content of *HSP18.2-ipt* plants (H lines) (A) and *SAG12-ipt* plants (S lines) (B) in comparison to the null transformant (NT) and wild type 'Penncross' (WT) of creeping bentgrass exposed to drought stress. The horizontal dashed line represents the average chlorophyll content of all plant lines under well-watered conditions as transgenic lines, WT, and NT did not differ in this treatment. The vertical bars indicate LSD values ($P \leq 0.05$) for comparison between plant lines at a given of drought stress where significant differences were detected.

Figure 5. Photochemical efficiency (Fv/Fm) of *HSP18.2-ipt* plants (H lines) (A) and *SAG12*-ipt plants (S lines) (B) in comparison to the null transformant (NT) and wild type 'Penncross' (WT) lines of creeping bentgrass. The horizontal dashed line represents the average Fv/Fm vlaue of all plant lines under well-watered conditions as transgenic lines, WT, and NT did not differ in this treatment. The vertical bars indicate LSD values ($P \le 0.05$) for comparison between plant lines at a given of drought stress where significant differences were detected.
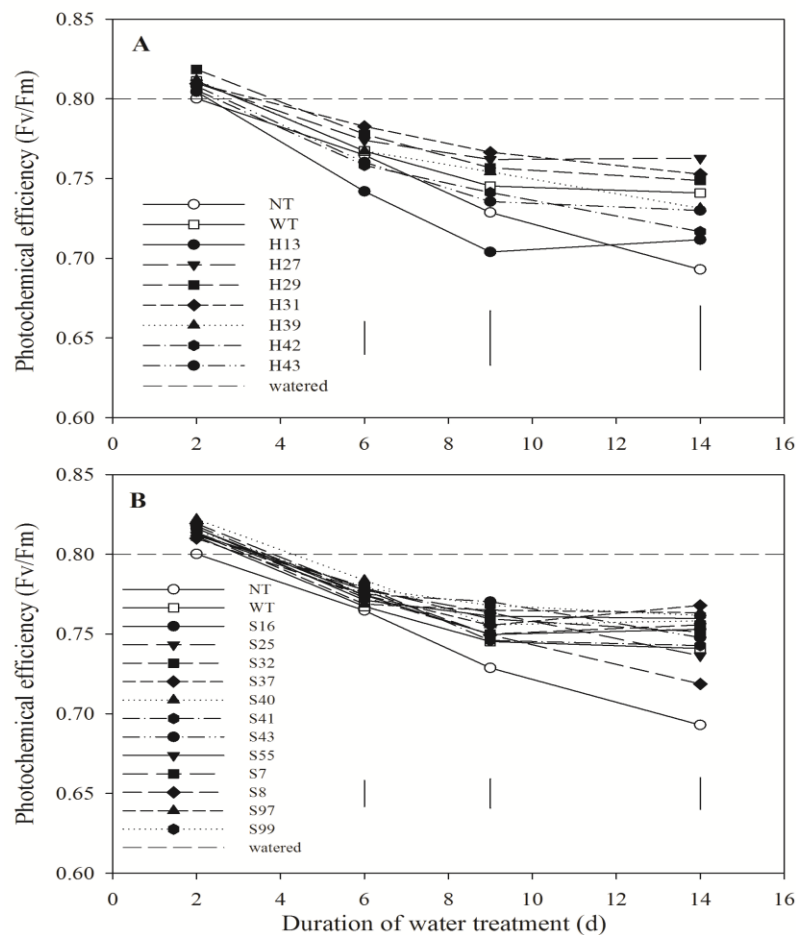
Figure 6.. (A) Total root biomass, (B) total root length, and (C) root:shoot ratios of the null transformant (NT), wild type 'Penncross' (WT), *HSP18.2-ipt* plants (H lines) and *SAG12-ipt* lines (S lines) of creeping bentgrass at 14 d of drought stress. The horizontal dashed line represents the average value of each parameter for all plant lines under well-watered conditions as transgenic lines, WT, and NT did not differ in this treatment. The vertical bar indicates LSD value ($P \leq 0.05$) for comparison between plant lines at 14 d of drought stress. Columns marked with an asterisk indicate plant lines exhibiting significant differences from the WT plants.
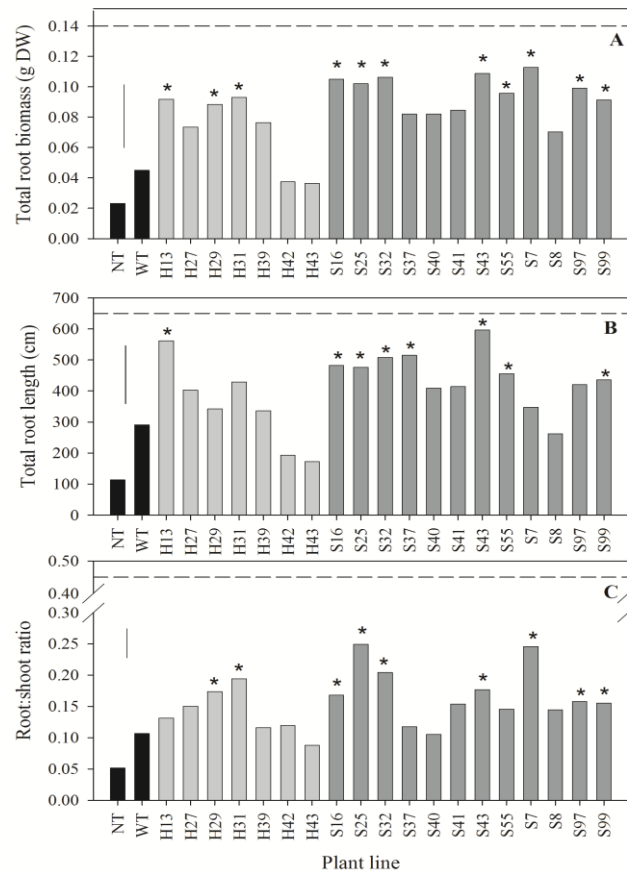
Figure 7. (A) isopentyl adenine (iPA) content and (B) abscisic acid (ABA) content in leaves of the null transformant (NT), wild type 'Penncross' (WT), *HSP18.2-ipt* plants (H lines) and (B) *SAG12-ipt* plants (S lines) of creeping bentgrass at 14 d of drought stress. The horizontal dashed line represents the average value of each parameter for all plant lines under well-watered conditions as transgenic lines, WT, and NT did not differ in this treatment. The vertical bar indicates LSD value ($P \leq 0.05$) for comparison between plant lines at 14 d of drought stress. Columns marked with an asterisk indicate plant lines exhibiting significant differences from the WT plants.
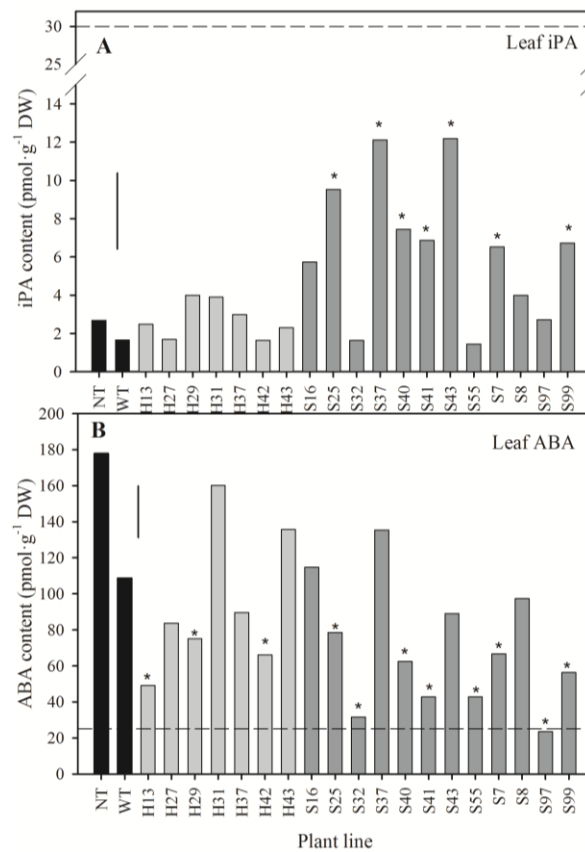
Figure 8. (A) isopentyl adenine (iPA) content and (B) abscisic acid (ABA)

content in roots of the null transformant (NT), wild type 'Penncross' (WT),

HSP18.2-ipt plants (H lines) and (B) *SAG12-ipt* plants (S lines) of creeping

bentgrass at 14 d of drought stress. The horizontal dashed line represents the

average value of each parameter for all plant lines under well-watered conditions

as transgenic lines, WT, and NT did not differ in this treatment. The vertical bar

indicates LSD value ($P \leq 0.05$) for comparison between plant lines at 14 d of

drought stress. Columns marked with an asterisk indicate plant lines exhibiting

significant differences from the WT plants.
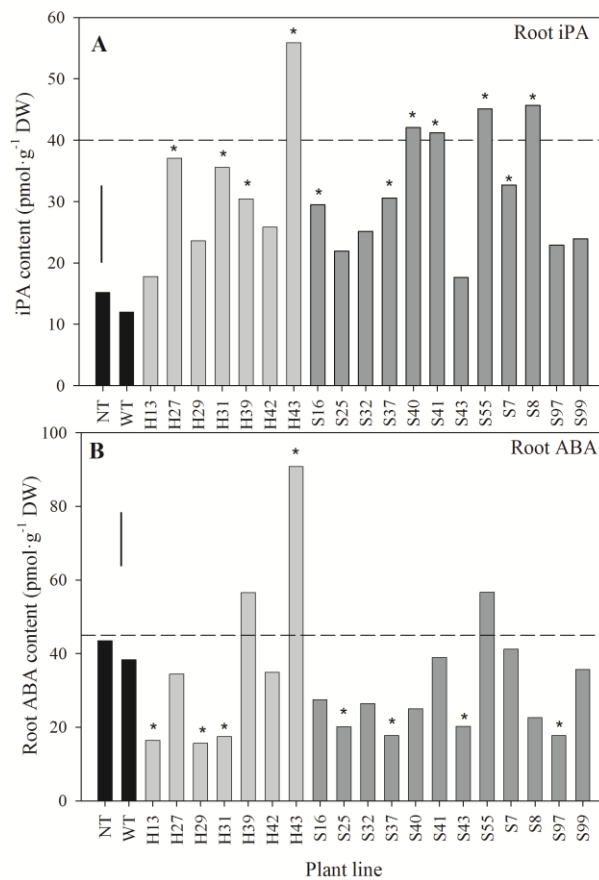
CHAPTER 2

PHOTOSYNTHESIS, WATER USE, AND ROOT VIABILITY

UNDER WATER STRESS AS AFFECTED BY EXPRESSION OF SAG12-

IPT CONTROLLING CYTOKININ SYNTHESIS IN AGROSTIS

STOLONIFERA

INTRODUCTION

A decline in water quality or availability for irrigation frequently disrupts the osmotic environment of the root zone and can lead to whole-plant water or osmotic stress. Typical symptoms of water stress include stomatal closure,leaf desiccation, leaf senescence, inhibition of photosynthesis, growth restriction, and root death, as well as other overall plant stress resistance mechanisms. Physiological damage caused by water stress and stress signaling are closely associated with the endogenous level and balance of hormones (Davies et al., 1994; Yang et al., 2002). Cytokinin (CK) synthesis and transport are typically inhibited whereas degradation is promoted under water stress, all of which have been associated with growth inhibition and a decline in stress tolerance (Yang et al., 2002; Kudoyarova et al., 2006). It is well accepted that natural or stress-induced leaf senescence is related to a decline in CK content in various plant species (Naqvi, 1995). CK involvement in delaying leaf senescence has been shown by the exogenous application of CK (Richmond and Lang, 1957; Badenoch-Jones et al., 1996; Liu and Huang, 2002; Okamoto et al., 2010) and by increasing endogenous production of CK through transgenic modification of CK biosynthesis genes or genes regulating CK degradation pathways (Naqvi, 1995).

The CK biosynthesis gene *ipt* encodes for the enzyme isopentyl transferase, which catalyses the rate-limiting first step in *de novo* CK biosynthesis and promotes the formation of isopentenyladenosine-5'-monophosphate (iPa) (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984; McGraw, 1987). The different forms of

endogenous *ipt* genes are expressed at relatively low levels in the control

condition and during drought stress in several plant species and are highly organ,

developmental, or cell type-specific and are down-regulated during stress

(Vyroubalova *et al.*, 2009). Generally, it has been demonstrated that *ipt*

expression increases endogenous CK or maintains CK levels under stress

conditions, thereby delaying leaf senescence and promoting stress resistance in

several plant species, such as *Arabidopsis* (*Arabidopsis thaliana*) (Medford *et al.*,

1989; Zhang *et al.*, 2000), lettuce (*Lactuca sativa*) (McCabe *et al.*, 2001), tobacco

(*Nicotiana tabacum*) (Rivero *et al.*, 2007), petunia (*Petunia*×*hybrida*) (Clarke *et

al.*, 2004), tall fescue (*Festuca arundinacea*) (Hu *et al.*, 2005), and creeping

bentgrass (*Agrostis stolonifera*) (Xu *et al.*, 2009; Merewitz *et al.*, 2010).

However, the physiological mechanisms of CK regulation of plant tolerance to

water stress remain less well-documented than other water stress regulators such

as abscisic acid (Bray, 1993; Kramer and Boyle, 1995; Chaves *et al.*, 2003;

Marrion-Poll and Leung, 2006). Analysis of the various organs of different

developmental stages of *ipt* transgenic plants with different growth habits and

stress resistance mechanisms is critical for further the documentation of the

effects of CK on water stress tolerance and the effects of water stress on CK

changes.

Previous studies have used senescence activated promoters, such as

SAG12 and *SARK* to auto-regulate or control *ipt* expression to prevent over-

production of CK, which may occur with constitutive expression (Medford *et al.*,

1989; Gan and Amasino 1995; Morris, 1995; Rivero *et al.*, 2007; Verdonk, *et al.*,

2008). Transformation of tobacco (*Nicotiana tabacum*) with *ipt*, regulated by a senescence-inducible promoter, resulted in significant improvement in drought tolerance, attributed to the delay in leaf senescence and increases in photosynthesis rates and antioxidant activities (Rivero *et al.*, 2007, 2009). Leaf senescence of mature leaves may be a mechanism for drought survival in order to reduce the surface area for transpiration and to redirect energy reserves to reproductive systems in annual crops where a high yield of the reproductive organs is desirable (Chaves, 2003). However, the maintenance of older leaves by the avoidance of senescence can be beneficial for additional energy produced by maintaining a greater amount of photosynthetic leaves as was found in tobacco (Rivero *et al.*, 2007). The much reduced growth rate of mature leaves largely prevents them from being a sink to draw nutrients and energy from immature leaves or roots that could alternately have gone towards drought survival (Khan, 1981).

For perennial grasses, delaying leaf senescence and maintaining physiological function of both immature and mature leaves under water stress is important for plant biomass production in forage and for the aesthetic appearance in turfgrasses and may improve drought tolerance. Previously, it has been demonstrated that expression of *ipt* in 11 creeping bentgrass transgenic lines maintained higher levels of CK, increased leaf chlorophyll content (Chl), and higher root to shoot ratios after 14 d of drought stress relative to the WT (Merewitz *et al.*, 2010). Cytokinins also regulate many other processes, including stomatal opening, photosynthesis, water relations, and root growth. However, how

elevated CK in a perennial grass species such as creeping bentgrass may alter

those processes related to water stress tolerance is not clear. Furthermore, limited

information is available about the root growth characteristics of *ipt* plants, which

is an important factor influencing water uptake under water stress. In the current

study, physiological responses of a typical *SAG12-ipt* transgenic line (S41) that

exhibited superior drought tolerance compared with the wild-type 'Penncross' of

creeping bentgrass were examined in an attempt to elucidate the physiological

changes associated with increased CK in the *SAG12-ipt* transgenic plants in both

the shoots and roots under water stress. The specific objectives of the study were

to determine *ipt* gene expression patterns in leaves and roots during the

progression of water stress and to examine the physiological effects of *ipt*

expression on immature and mature leaves and in roots for creeping bentgrass

exposed to water stress. Physiological assessment focused on leaf senescence

(chlorophyll content), water relations (relative water content, osmotic adjustment,

stomatal conductance, transpiration, and water use efficiency), and photosynthetic

activities (photochemical efficiency and net photosynthetic rate). In addition,

since enhanced rooting in the *SAG12-ipt* plants was previously reported, the aim

here was therefore to evaluate whether the enhanced rooting was due to delayed

root senescence or increased root viability.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Transgenic creeping bentgrass plants were produced by the *Agrobacterium tumefaciens* transformation method as described previously (Merewitz *et al.*, 2010; Xu *et al.*, 2009; Xing *et al.*, 2010). Plant material included the wild-type cultivar 'Penncross' (WT) and the *SAG12-ipt* transgenic line (S41) which performed well under drought stress in our previous drought study (Merewitz *et al.*, 2010). A hydroponic growth method was used for uniformity of water stress imposition in order to reduce the potential confounding effects of nutrient deficiencies and to eliminate root damage during sampling, which may occur with soil-based water stress imposition. All plant lines were grown in a hydroponic system within a large walk-in controlled environment growth chamber with conditions set to maintain a 12 h photoperiod, 50% relative humidity, 500 μmol m^{-2} s^{-1} photosynthetic photon flux density, and a day/night temperature of 23/20 °C. Stock solutions (1000×) of the following nutrient solutions were prepared and diluted into Hoagland's nutrient solution: ammonium sulphate ((NH_4)$_2SO_4$, 71.361 g l^{-1}), potassium nitrate (KNO_3, 27.3 g l^{-1}), calcium nitrate ($Ca(NO_3)_2.4H_2O$, 127.521 g l^{-1}), potassium phosphate (KH_2PO_4, 68.045 g l^{-1}), potassium sulphate (K_2SO_4, 43.568 g^{-1}), magnesium sulphate ($MgSO_4.7H_2O$, 199.65 g l^{-1}), disodium ethylenediaminetetraacetate (Fe(EDTA)Na, 14.684 g l^{-1}), Micronutrients (H_3BO_3, 1.43 g l^{-1}, $MnCl_2.4H_2O$, 0.91 g l^{-1}, $ZnSO_4.H_2O$, 0.11 g l^{-1}, $CuSO_4$, 0.04 g^{-1}, (NH_4)$Mo_7 O_{24}.4H_2O$, 0.01 g l^{-1}). Plants were suspended within 1.27 cm holes in Styrofoam boards that fit within plastic bins (54×42×14

cm in height) that floated on the growth media. The hydroponic solution was aerated with a tube inserted into the solution through the Styrofoam connected to a pump (115 V, 60 Hz, Tetra® Blacksburg, VA). Solution pH was monitored and adjusted as needed every other day and the solution was changed on a weekly basis. Plants were not clipped to allow for adequate stem development for the separation of new and old leaves.

PEG-induced Water Stress

The osmotic potential of the growth solution was decreased in a stepwise manner for the imposition of water stress by weekly additions of increasing volumes of polyethylene glycol 8000 (PEG-8000). This chemical has been used in previous studies to impose water stress to plants and the large MW of 8000 to prevent root uptake of PEG (Lagerwerff *et al.*, 1961; Janes, 1974). The nutrient solution osmotic potential was approximately –0.05 MPa due to the presence of the nutrient salts. PEG-8000 was added to bring the osmotic potential of the solution to approximately –0.3, –0.5, –0.7, –1.0, and –1.4 MPa. The osmotic potential of the growing solution was monitored by running a sample of the solution into a vapour pressure osmometer (Vapro5520, Wescor, Inc. Logan, UT) after each addition of PEG-8000 was fully dissolved and at each treatment level as described in Burlyn (1983).

Physiological Analysis

Leaves were separated based on relative stem position, with the youngest two leaves considered immature and the remaining leaves considered mature.

Grass plants were hand trimmed twice a week prior to PEG treatment. Approximately 10 d before PEG treatment the plants were left uncut to allow for more vertical growth for the separation of immature and mature leaves. The plants were not trimmed during the PEG treatment. Overall turf performance was evaluated by visually rating turf quality (TQ). Turf quality was visually rated every 2 d based on leaf wilting, turf uniformity, colour, and density on a scale of 1 to 9 with 1 being brown and desiccated turf, 6 being the minimal acceptable level, and 9 being a green and dense turf (Turgeon, 2008).

Relative water content (*RWC*) of leaves was measured as an indicator of leaf hydration status. Leaf *RWC* was calculated based on fresh (*FW*), turgid (*TW*), and dry weights (*DW*) of approximately 0.1 g of leaf samples using the following formula: $(FW–DW)/(TW–DW)\times100$. Leaf *FW* was determined on a mass balance immediately after being excised from the plants. Turgid weights were determined after soaking the leaves in deionized water for 12 h in a closed Petri dish at 4 °C and weighed immediately after being blotted dry. Leaves were then dried in an 80 °C oven for at least 72 h prior to weighing for DW (Barrs and Weatherley, 1962).

Leaf chlorophyll content (Chl) and photochemical efficiency (F_v/F_m) were measured to evaluate leaf senescence. Total Chl was extracted in the dark for 72 h in dimethyl sulphoxide. The absorbance of the leaf extract was measured at 663 nm and 645 nm with a spectrophotometer (Spectronic Genesys 2; Spectronic Instruments, Rochester, NY, USA). Chl was calculated using the formula described in Arnon (1949). F_v/F_m was evaluated as a ratio of the variable

fluorescence (F_v) to the maximal fluorescence (F_m) value determined using a chlorophyll fluorescence meter (Fim 1500; Dynamax, Houston, TX, USA). Leaf clips were used to adapt individual leaves to darkness for 30 min prior to reading the F_v/F_m ratio with the fluorescence meter. Two subsamples were taken per plant at each sampling day.

Osmotic adjustment (*OA*) was determined by measuring the osmotic potential of leaf sap of fully rehydrated leaves. Leaves samples were allowed to soak in deionized water overnight, blotted dry, placed into micro-centrifuge tubes, frozen in liquid nitrogen, and stored at $-20\ ^\circ$C until further analysis. Thawed leaves were then immediately ground with a micro-pestle to express the leaf sap. The sap was then inserted into osmometer (Wescor, Inc., Logan, UT) for the determination of the osmolality (mmol kg^{-1}). Osmolality was converted to osmotic potential and *OA* was then calculated as the difference between the well-watered control and drought-stressed plants (Blum, 1988).

Roots were washed free of PEG nutrient solution for CK extraction and root viability measurements. Root CK content was determined in the same way as for leaves, but were bulked due to difficulties in separating younger and older roots. Root viability was estimated by measuring the activity of dehydrogenase by using the triphenyltetrazolium chloride (TTC) reduction technique (Knievel, 1973; McMichael and Burke, 1994). The activity was based on the dry weight of the root sample, determined after drying in an 80 $^\circ$C oven for at least 72 h.

Net photosynthetic rate (*Pn*), stomatal conductance (g_s), and transpiration rate (*E*) were measured using a leaf chamber (6 cm^2) of an infrared gas exchange analyser (Li-Cor 6400, Li-Cor, Inc., Lincoln, NE). Detached leaf samples of approximately 5–10 leaves were immediately placed in the leaf chamber, which provided red and blue light from an LED source (665 nm and 470 nm ranges), 400 μl l^{-1} CO_2. Leaf area of the *Pn* sample was measured with Digimizer software (MedCalc Software bvba; Mariakerke, Belgium) using scanned digital images of the fresh leaf samples. *Pn*, g_s, and *E* values were converted from the 6 cm^2 area of the leaf chamber to the actual *Pn*, g_s, and *E* values based on the measured leaf area. Instantaneous water use efficiency (*WUE*) was calculated as a molar ratio of *Pn* to *E* (μmol CO_2 m^{-2} s^{-1} mmol^{-1} H_2O m^{-2} s^{-1}).

Carbon isotope discrimination (Δ) analysis has been shown to be negatively correlated to *WUE*, and used widely to estimate *WUE* in various plant species (Johnson and Basset, 1991). The Δ value was measured as described previously (DaCosta and Huang, 2006). Briefly, leaf tissue samples were separated based on leaf position and maturity, ground to a fine powder, passed through a 45-mesh screen, and sent to the Stable Isotope Ratio Facility for Environmental Research (University of Utah, Salt Lake City) for the measurement of carbon isotopic composition (Smedley *et al.*, 1991; Ebdon *et al.*, 1998). The Δ (per mil ‰) value was calculated using the equation ($\delta a - \delta p$)/($1 + \delta p$) where δp was the C isotopic composition of the plant sample and $\delta a = 28‰$ the standard C isotopic composition of the source air (Farquhar and Richards, 1984; Farquhar *et al.*, 1989).

Endogenous CK content was measured by extraction and quantification by an indirect enzyme linked immunosorbent assay (ELISA) method described in Setter *et al.* (2001) with modifications (Wang *et al.*, 2003). Samples were extracted in 80% (v/v) methanol and purified with reverse phase C_{18} columns. Hydrophilic contaminants were removed and the CK were eluted with 30% methanol. Total CK was calculated as the sum of the contents of iPa, zeatin riboside (ZR), and dihydrozeatin riboside (DHZR).

Gene Expression Analysis

Total RNA was extracted from leaves using the RNeasy Plant Mini Kit method according to the manufacturer's protocol (Qiagen Inc., Valencia, CA). The DNA-free protocol was used prior to reverse transcription-polymerase chain reaction (RT-PCR) analysis to eliminate contamination (Am1906, Ambion, Inc., Austin, TX). Gel electrophoresis and absorbance at 260/280 nm was performed to ensure RNA integrity. RT-PCR was performed on 2 μg of each RNA extract on illustra ready-to go RT-PCR beads (27-9266-01, GE Healthcare, Piscataway, NJ) with a *Taq* polymerase enzyme (R001-A, Takara, Madison, WI) set to program rt-pcr50 of the GeneAmp PCR system (9700, Applied Biosystems, Inc., Foster City, CA). PCR control was used to test for contamination.

Experimental Design and Statistical Analysis

The experimental design was a split-plot design with irrigation treatment as the main plots and plant materials as the sub-plots, with four replicates for each irrigation treatment and grass material. Effects of watering treatment, plant

materials, and corresponding interactions were determined by analysis of variance

according to the general linear model procedure of SAS (Version 9.0; SAS

Institute, Cary, NC). Differences between watering treatments and plant means

were separated by Fisher's protected least significance difference (LSD) test at the

0.05 probability level.

RESULTS

RT-PCR Analysis of *ipt* Expression

No expression of *ipt* was detected in the WT line in either leaves or roots. The expression of *ipt* was detected in immature leaves of *SAG12-ipt* transgenic plants in all PEG treatments, and the transcript level remained relative constant throughout the PEG treatments; *ipt* was detected in transgenic plants without PEG treatment (at 0 MPa) (Fig. 1). In mature leaves of transgenic plants, there was a gradual increase in *ipt* transcript abundance in PEG treatments from 0 to –0.7 MPa and then a decline at higher level of PEG-induced water stress (–1.4 MPa). The *ipt* expression was detected in roots of *SAG12-ipt* plants in all treatments and transcript abundance increased with increasing levels of PEG-induced water stress, with the *ipt* transcript levels approximately three times higher at –1.4 MPa than at 0 MPa.

CK Content of Immature Leaves

The iPA content in immature leaves was significantly higher in *SAG12-ipt* plants than in the WT at 0 MPa and during the PEG treatments (Fig. 2). The iPA content declined at –1.4 MPa in all plants, but to a greater extent in WT (65%) than in *SAG12-ipt* plants (24%) (Fig. 2A). DHZR content declined significantly in both WT and transgenic plants during PEG treatment (Fig. 2B). A significantly higher DHZR content in the transgenic plants was detected only at –0.7 MPa, which was 7% higher than in the WT. ZR content was not different between the WT and *SAG12-ipt* plants under the control conditions or throughout the duration

of PEG treatment (Fig. 2C). PEG-induced drought stress reduced the ZR content of immature leaves by an average of 71% for both lines. Total CK content (including iPA, ZR, and DHZR) did not differ between the plant lines at the control (0 MPa) level, however, after the osmotic potential of the growth solution was reduced from −0.7 to −1.4 MPa, significant differences were observed. For instance, at −0.7 and −1.4 MPa, total CK in WT plants was reduced by 45% and 64%, respectively, whereas that in the transgenic plants was reduced by 14% and 52%, respectively (Fig. 2D).

CK Content of Mature Leaves

iPA content of mature leaves was greater in the transgenic plants compared to WT at all levels of PEG treatment (Fig. 3A). iPA content declined with PEG-induced water stress, but the rate of iPA loss was slower in the *SAG12-ipt* plants than in WT. For instance, at −1.4 MPa iPA declined by 57% and 13% relative to their respective control level in WT and *SAG12-ipt* plants, respectively. Differences in DHZR content between plant lines were detected only at −1.4 MPa, which was 10% greater in the transgenic plants than the WT (Fig. 3B). ZR content in mature leaves was not statistically different between plant lines at any PEG treatment levels (Fig. 3C). Total CK in mature leaves did not differ between plant lines in the control condition, but was maintained at a significantly higher level in the transgenic plants than in the WT at −0.7, −1.0, and −1.4 MPa, which was approximately 25% higher level at −1.4 MPa (Fig. 3D).

Leaf Water Status

RWC of both immature and mature leaves did not differ between plant lines under the control conditions, but decreased with increasing severity of PEG-induced drought stress (Fig. 4). The transgenic plants maintained significantly higher RWC than WT in immature leaves at −1.4 MPa and in mature leaves at −1.0 and −1.4 MPa. Osmotic adjustment (*OA*) increased with increasing levels of PEG-induced water stress and was significantly higher in *SAG12-ipt* plants than in the WT in both immature and mature leaves in most of the PEG treatments (Fig. 5).

Photosynthetic Parameters and Carbon Isotope Discrimination

The transgenic line and WT did not differ for Chl content in both immature and mature leaves under the control conditions (Fig. 6). Chl content was significantly greater in the transgenic plants than the WT at −1.4 MPa for immature leaves and under all levels of PEG-induced water stress for the mature leaves.

Leaf photochemical efficiency (F_vF_m) declined in both immature and mature leaves in response to increasing severity of PEG-induced water stress (Fig. 7). Immature leaf F_v/F_m did not differ significantly between the WT and *SAG12-ipt* plants under control or PEG treatments. In mature leaves, F_vF_m was significantly higher in *SAG12-ipt* plants from −0.5 to −1.4 MPa during the PEG treatment (Fig. 7).

Plant lines differed in net photosynthetic rate (Pn), stomatal conductance (g_s), transpiration rate (E), and water use efficiency (*WUE*, calculated as Pn/E ratio) of both immature and mature leaves at −0.7 and −1.0 MPa, but not in the control or other PEG treatment levels (Table 1). Values for *Pn* were significantly higher for the transgenic plants compared to the WT at −0.7 and −1.0 in immature leaves, and at −0.7 MPa for mature leaves. Mature leaves of transgenic plants had higher g_s than the WT at the −0.7 MPa treatment, but did not differ from the WT for transpiration rate. The *WUE* of transgenic plants were significantly greater than the WT for immature leaves at −0.7 MPa and for mature leaves at −0.7 and −1.0 MPa. In response to PEG treatment, carbon discrimination ratio (Δ) declined in both WT and transgenic plants, however, transgenic plants had lower Δ values in immature leaves at −1.4 MPa and in mature leaves at −1.0 and −1.4 MPa treatment (Fig. 8).

CK Content of Roots and Root Viability

Root iPa content increased in response to PEG treatment to approximately 400% of the control level in transgenic plants and 100% in the WT at −1.4 MPa (Fig. 9). Roots of transgenic plants were significantly higher than the WT roots under control and PEG treatment, and the difference was most pronounced at −1.4 MPa. Root DHZR content was unchanged during PEG treatment and no significant differences in DHZR content were detected between the WT and transgenic plants. ZR content of the transgenic plants was significantly higher at −

1.0 and -1.4 MPa, as ZR content of the WT declined to zero in these treatments while it was maintained in the transgenic plants.

Root viability determined by TTC reduction decreased relative to the control level in response to PEG treatment in both plant lines (Fig. 10). The reduction in root viability was greater for the WT plants, since the average rate of TTC reduction was reduced to 40% of the control level in the WT and by approximately 25% in the transgenic plants at -1.4 MPa. Root viability did not differ between the plant lines under the control conditions, but were significantly greater in the transgenic plants than the WT during PEG treatment.

DISCUSION

PEG-induced water stress through lowering the osmotic potential of the growth solution (from 0 to −1.4 MPa) caused physiological damage in both the leaves and roots of the WT and *SAG12-ipt* transgenic plants; however, the transgenic plants exhibited better tolerance to the PEG-induced water stress, as demonstrated by the maintenance of higher F_vF_m, Chl content, *RWC*, *Pn*, g_s, and CK content, particularly in mature leaves, and the greater viability and CK production of roots. The same transgenic line has previously been reported to exhibit superior drought tolerance compared with the WT when plants were subjected to soil drying by withholding irrigation (Merewitz *et al.*, 2010). The results, in combination with our previous study, demonstrated that expression of the *ipt* gene in creeping bentgrass was effective in improving plant tolerance to water stress.

The expression of *ipt* was detected in immature and mature leaves as well as in roots under all levels of PEG-induced water stress in transgenic creeping bentgrass. PEG-induced water stress could have evoked an initial senescence response, which activated the *SAG12* promoter for expression of *ipt* in immature and mature leaves and roots of *SAG12-ipt* plants. Drought has been shown to cause expression of *ipt* in a previous study (Clarke *et al.*, 2004). Expression of *SAG12-ipt* was also detected under non-stressed conditions in this study and similar findings were reported previously in other plant species, such as in petunia (*Petunia×hybrida*) (Clarke *et al.* 2004), maize (*Zea mays*) (Robson *et al.*, 2004),

and tobacco (Rivero *et al.*, 2007), which has been attributed to natural senescence-induced expression. Expression under non-stressed conditions has also been attributed to expression of the endogenous *ipt* genes, since several *ipt* genes have recently been isolated from species such as *Arabidopsis* (Sakamoto *et al.*, 2006), petunia (*sho* gene; Zubko *et al.*, 2002), rice (*Oryza sativa* L., 10 forms of *ipt*; Sakamoto *et al.*, 2006), and maize (*ipt*1, *ipt*2, *ipt*4–8; Brugière *et al.*, 2008). Presumptively, the high level of *ipt* expression observed here under control conditions and in immature leaves of creeping bentgrass could be due to a combination of these factors; however, it seems more likely that it was induced by natural senescence since perennial grass leaves have a relatively high rate of leaf turnover due to the perennial growth habit.

The expression of *ipt* under different levels of PEG-induced water stress was associated with the elevated total CK content in immature and mature leaves and roots of *SAG12-ipt* plants relative to the WT plants. The enhancement of total CK accumulation was primarily due to increased levels of iPa, which is expected since the immediate end-product of the reaction catalysed by *ipt* is iPA (Medford *et al.*, 1989). In immature leaves, the higher *ipt* expression level was generally associated with higher levels of iPa and total CK content. Conversely, mature leaves had relatively low *ipt* expression in the non-stressed condition in conjunction with high CK content but during moderate to severe drought stress, higher expression was accompanied by moderate to low levels of CK. Thus, it is worth noting that *ipt* expression was not always closely correlated to CK content most likely due to drought damage or other factors affecting post-transcription

processes and CK abundance. The results suggest that there may be differences in auto-regulation of the *SAG12* promoter and post-trancription processes such as those regulating post-transcription modifications, transcript stability, and translation rates between immature and mature leaves. In addition, CK abundance caused by differences in CK destination, transport activity, and CK degradation due to cellular drought damage may contribute to this CK accumulation difference relative to expression levels between immature and mature leaves. The abundance and activities of cytokinin oxidase (Motyka *et al.*, 1996) and antioxidant enzymes (Synkova *et al.*, 2006) may contribute to these differences, since iPa is the preferred substrate for cytokinin oxidase (Redig *et al.*, 1997) and *ipt* plants have exhibited differential elevation of antioxidant transcripts and enzyme activity in different plant organs of tobacco (Rivero *et al.*, 2007). However, more research is needed to elucidate post-transcriptional phenomenon of *ipt* and how it associates with CK content between immature and mature leaves of creeping bentgrass.

The mechanisms of CK regulation of drought tolerance are not yet fully clear. It is known that CK stimulates stomatal opening, which could help with carbon absorption for photosynthesis, but may cause leaf desiccation under drought stress due to water loss (Chernya'dev, 2005). More recently, it has been found that the timing of increased CK content, the form of CK present, and the balance of hormones may be more critical in determining stomatal responses during drought stress, particularly with *ipt* plants (Pospisilova *et al.*, 2000, 2005). In the current study, immature leaves of the transgenic plants had higher *Pn* under

moderate levels of PEG-induced water stress (–0.7 and –1.0) but g_s and E did not

differ from the WT plants. In addition, mature leaves of the transgenic plants had

a lower Δ value relative to the WT throughout drought treatment at –0.5, –0.7,

and –1.4 MPa. iPa content, total CK content, and Pn were generally higher

compared with the WT during the PEG treatment, but there were few differences

in g_s or E in leaves of transgenic plants; the elevated CK may have affected Pn

through other mechanisms besides stomatal regulation. Similar results were found

in water-stressed ipt tobacco since stomatal conductance was largely the same

between ipt and WT plants (Rivero $et\ al.$, 2009). They concluded that

maintenance of photosynthesis rates in ipt tobacco was possibly due to non-

stomatal traits such as increased photorespiration, which promotes photosynthesis

under drought stress by providing RUBP and other beneficial metabolites

(Wingler $et\ al.$, 2000). This was evident to Rivero $et\ al.$ (2009) by the increased

level of transcripts coding for enzymes in the photorespiration pathway, increased

metabolites generated by photorespiration, greater antioxidant content, and an

increase in the CO_2 compensation point in ipt plants compared with the WT.

These mechanisms may also be a factor in $SAG12\text{-}ipt$ creeping bentgrass observed

indirectly in this study as higher Pn in the mature leaves of the transgenic plants,

increases in chlorophyll content, and greater F_v/F_m. In addition, differences in Pn

contributed to differences in WUE between plants lines. This is in conjunction

with the differences in Δ values between the WT and transgenic plants, as

transgenic plants had lower Δ than the WT during most of the PEG treatments. In

Kentucky bluegrass ($Poa\ pratensis$), higher values of WUE have been associated

with less wilting, greater TQ, and superior water relations under drought stress (Ebdon and Kopp, 2004). In several cool season grasses, low Δ was associated with higher instantaneous water use efficiency; plants with low Δ had higher water potential, solute potential, and turgor pressure, exhibiting a better capacity for growth under drought stress (Johnson and Basset, 1991). Thus, most likely the *ipt* gene and increased CK may enhance metabolic activities that may promote photosynthetic activity without increasing stomatal conductance or transpiration rate, thereby increasing water use efficiency, especially in immature leaves. CK involvement in regulating *Pn*, g_s, *E*, *WUE*, and Δ under drought stress deserves additional investigation since increases in CK is not always associated with increases in g_s as previously proposed and some studies have shown a decrease in g_s in response to elevated CK (Pospisilova *et al.*, 1997, 1998).

Our results show that CK may have some involvement in regulating water relations and OA, as *SAG12-ipt* plants had better *OA* and retained more water, particularly in mature leaves. However, it is not clear whether this is a direct, primary result of altered *OA* and water status or a secondary effect of improved water status due to increased overall physiological activities or photosynthetic efficiency. Recent literature in the role of CK in regulating osmotic adjustment is conflicting. In transgenic potato that accumulate CKs (*Solanum tuberosum*), *RWC* and *OA* seemed to be unaffected by CK levels (Siffel *et al.*, 1992; Popsisilova *et al.*, 2000). Conversely, *ipt* plants have also been shown to have enhanced levels of osmolytes such as proline, which would typically have an effect on OA. The changes in proline in this study were not always associated with reduced wilting

of *ipt* plants (Thomas *et al.*, 1995). Thus, due to the complexities of comparing different plant species under different promoters expressing *ipt*, a clear role of CK in regulating water relations is still not fully elucidated. It has been suggested that CK may cause a slight osmotic response similar to a small degree of salt stress to allow increased tolerance via adaption for subsequent stress such as drought (Thomas *et al.*, 1995; Pospisilova *et al.*, 2000). Our results seem to be consistent with this explanation since CK levels were higher and *OA* was increased even under a low level of water stress (–0.3 MPa), and, consistently, the transgenic plants, compared to WT, in both immature and mature leaves, even when differences in other parameters such as *RWC* or F_v/F_m were not yet observed.

Effects of *ipt* expression on the physiological responses to PEG-induced water stress varied between mature and immature leaves. Generally, the positive effects of *ipt* expression on the physiological parameters examined here were more pronounced for mature leaves, whereas fewer differences occurred for immature leaves between the transgenic and WT plants. Significant differences between *SAG12-ipt* and WT in *Pn*, *WUE*, and Δ in immature leaves were observed earlier during moderate water stress (–0.7 and –1.0 MPa), whereas differences between *SAG12-ipt* and WT in Chl, F_vF_m, *E*, *RWC*, and *OA* in immature leaves, were not observed until more severe water stress levels were reached (–1.0 to –1.4 MPa). In mature leaves, differences between *SAG12-ipt* and WT in most of these parameters started at approximately –0.5 MPa. Senescence was delayed in mature leaves of *SAG12-ipt* plants compared with mature leaves of WT, indicated by maintenance of higher Chl and F_vF_m during water stress. Delay

in leaf senescence is significant for plants to adapt to water stress, as the first symptoms of senescence are seen as a loss of chlorophyll and chloroplasts as the plant degrades leaf mesophyll cells for nutrient remobilization (Lim *et al.*, 2007). The results of this study show that the expression of the *SAG12-ipt* in creeping bentgrass at highly detectable levels was sufficient to maintain levels of CK in immature and mature leaves and increase root CK of creeping bentgrass subjected to water stress. It may be assumed that enough CK was produced to overcome degradation of the free forms of CK by cytokinin oxidases, which are up-regulated in the drought response in most plant organs (Vyroubalova *et al.*, 2009).

Root *ipt* expression analysis revealed a gradual increase in transcript abundance with the severity of PEG-induced water stress, which corresponded to an increase in CK levels at -1.4 MPa osmotic potential. Despite the increase in *ipt* transcription during moderate (-0.5 to -1.0 MPa) water stress, a corresponding increase in root CK content during moderate stress was not observed, which could be due to sustained CK transport to the leaves. However, in roots exposed to -1.4 MPa PEG treatment, a sharp increase in CK was observed, which could be due to the interruption of CK transport from roots to other parts of a plant under severe water stress. A decline of root CK content during moderate water stress followed by an accumulation of CK in the roots during severe water stress was also found in *SAG12-ipt* tobacco (Havlova *et al.*, 2008). The results of the current study suggest that this could be due to reduced CK transport activity as opposed to a decline in root viability, as *SAG12-ipt* root viability was significantly higher at $-$1.4 MPa relative to the WT plants. Other work has also suggested that CK

accumulates in roots under severe drought stress due to reduced xylem transport of CK or possibly due to a decrease in CKX activity (Novakova *et al.*, 2007). Kudoyarova *et al.* (2006) reported that loading of CK into the xylem was decreased in tomato plants exposed to dry soils. Similar results were found in response to heat stress, *SAG12-ipt* bentgrass exhibited increased iPa in the roots by approximately ten times the amount in the roots at optimal temperature of 20 °C, whereas the leaves had a less dramatic increase in iPa content in response to temperature, however, leaf iPa content was maintained at higher levels in the transgenic lines compared with the WT. Similar to our results, a less dramatic change was observed for the other forms of CK such as ZR and DHZR compared with iPa in response to temperature and the transgene in this study (Xu *et al.*, 2009).

Limited information is available about the role of CK in root senescence and mortality under drought-stressed conditions. Our results indicate that increased endogenous CK in the *SAG12-ipt* plants was associated with higher root viability. The results show that CK may be involved in regulating root mortality, since *SAG12-ipt* plants had increased root viability under water-stress conditions. Promoting root growth and viability were previously reported in a study with exogenous applications of different forms of CKs on creeping bentgrass exposed to heat stress (Zhang and Ervin, 2008). The mechanisms for root survival of water stress associated with *ipt* expression are not known. It is possible that the delay in leaf senescence and the maintenance of higher photosynthesis of the *SAG12-ipt* plants under water stress could have maintained adequate carbon available to

transport to the roots to maintain root growth or survival. Alternatively, CK may directly be involved in the regulation of root mortality; however, this is not yet clear. Previous research has indicated CK may play a direct role in root stress signalling by stimulation of root antioxidants to reduce root lipid peroxidation, which increased root viability (Liu and Huang, 2002). Programmed cell death (PCD), primarily of root tips, is caused by abiotic stress such as drought and may be mediated by reactive oxygen species (ROS) accumulation that causes an endoplasmic reticulum (ER) signal to cause PCD (Duan *et al.*, 2010). Since *ipt* plants may maintain more antioxidants due to the presence of increased CK, there may be less of a signal sent to the ER, to reduce PCD of the roots under drought for increased root viability. Recent work by Vyroubalova *et al.* (2009) has more explicitly shown that CKs most likely do not have a direct function in drought signalling, since CK changes occur slowly and the reduced growth rate is not caused by decreased active CK or increased CK oxidation, but rather due to changes in abscisic acid. They concluded that enhanced CK content by transgenic methods may be the best method for the creation of cultivars with increased drought tolerance by promoting root growth under stress. Future work will analyse antioxidant enzyme responses in *SAG12-ipt* creeping bentgrass under drought stress. Our results indicate that increased endogenous CK may help delay drought-induced root mortality; however, the exact mechanism deserves more attention, especially the effects of increased CK on root tip viability under water stress.

In conclusion, *SAG12-ipt* transformation of creeping bentgrass resulted in increases in CK accumulation in the leaves and roots and in the overall plant tolerance to water stress. The physiological effects of *ipt* expression on improving plant tolerance to water stress were reflected by an enhancement in OA, photosynthetic characteristics, water use efficiency, delay in leaf senescence, and maintenance of higher root viability under water stress in creeping bentgrass. Future work will aim to identify the mechanisms associated with CK regulation of photosynthesis, OA, and root viability in *SAG12-ipt* creeping bentgrass during water stress. In addition, the interaction of CK derived from *SAG12-ipt* with other stress regulation hormones, such as ABA, auxin, and ethylene may deserve further investigation.

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TABLES AND FIGURES

Table 1 Net photosynthesis rate (Pn), stomatal conductance (g_s), Transpiration rate (E), and instantaneous water use efficiency (WUE) of immature and mature leaves of wild type 'Penncross' (WT) and *SAG12-ipt* (S41) plants exposed to PEG-induced drought stress via reduced osmotic potential of the growing solution (-0.7 and -1.0 MPa). Values followed by the same letter are not significantly different based on Fisher's protected LSD test ($P \leq 0.05$) between plant lines at a given osmotic potential.

Leaf age	MPa	Plant	Pn (CO_2 µmol m^{-2}s^{-1})		g_s (mmol)		E (mmol H_2O m^{-2} s^{-1})		WUE (µmol mmol^{-1})	
Immature	-0.7	WT	5.35	b	13.96	a	3.31	a	1.62	b
		S41	5.66	a	12.62	a	2.84	a	1.99	a
		LSD	0.29		0.70		1.00		0.31	
	-1.0	WT	1.41	b	2.05	a	0.71	a	1.98	a
		S41	1.74	a	2.93	a	0.79	a	2.21	a
		LSD	0.27		1.30		0.30		0.50	
Mature	-0.7	WT	2.83	b	6.32	b	2.26	a	1.25	b
		S41	3.82	a	8.52	a	2.37	a	1.61	a
		LSD	0.42		0.40		0.60		0.30	
	-1.0	WT	0.29	a	1.86	a	0.55	a	0.52	b
		S41	0.26	a	1.97	a	0.38	a	0.68	a
		LSD	0.31		0.18		0.29		0.15	

Figure 1. RT-PCR gel images (inset) and relative expression ratio (bar graph) of *ipt* to the actin internal control in immature leaves, mature leaves and roots of *SAG12-ipt* creeping bentgrass plants (line S41; lanes 2-8) and wild type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (0 to -1.4 MPa). C, PCR control, M, molecular weight marker, BP, base pair. Significance at $P \leq 0.05$ is indicated by mean standard error bars (n=3).
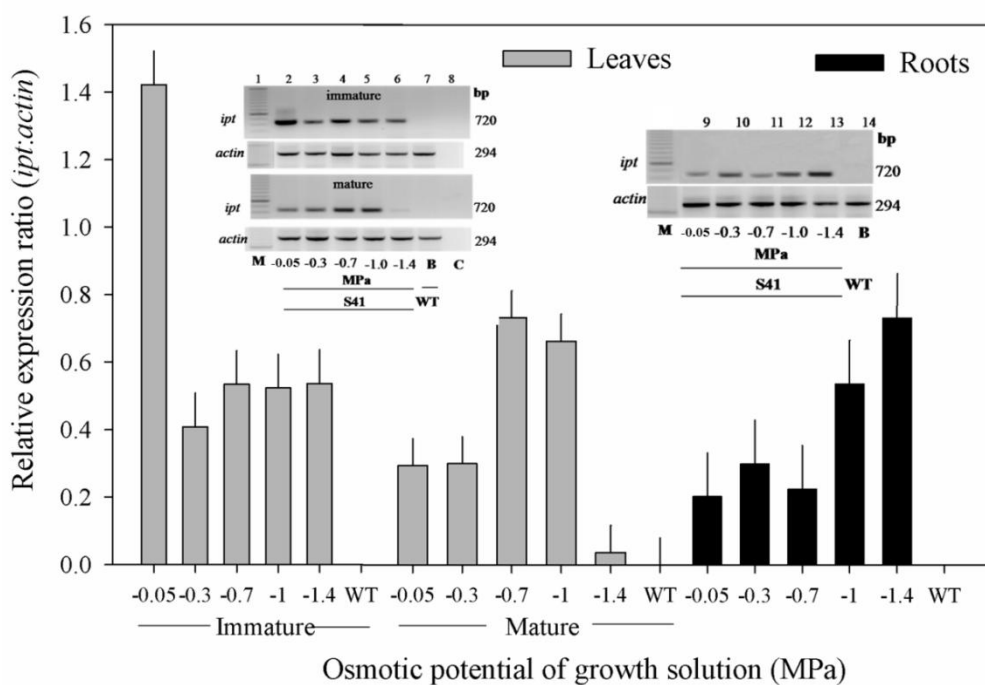
Figure 2. (A) Isopentyl adenine (iPa) (B) dihydrozeatin riboside (DHZR), (C) zeatin riboside (ZR) and (D) total cytokinin content (CK) (sum of iPa, DHZR, ZR) of immature leaves (top two leaves not fully expanded) of *SAG12-ipt* creeping bentgrass (line S41) and wild type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (0 to -1.4 MPa). Vertical bars indicate LSD values where significant differences were detected ($P \leq 0.05$) for comparison between plant lines at a given osmotic potential of the growth solution.
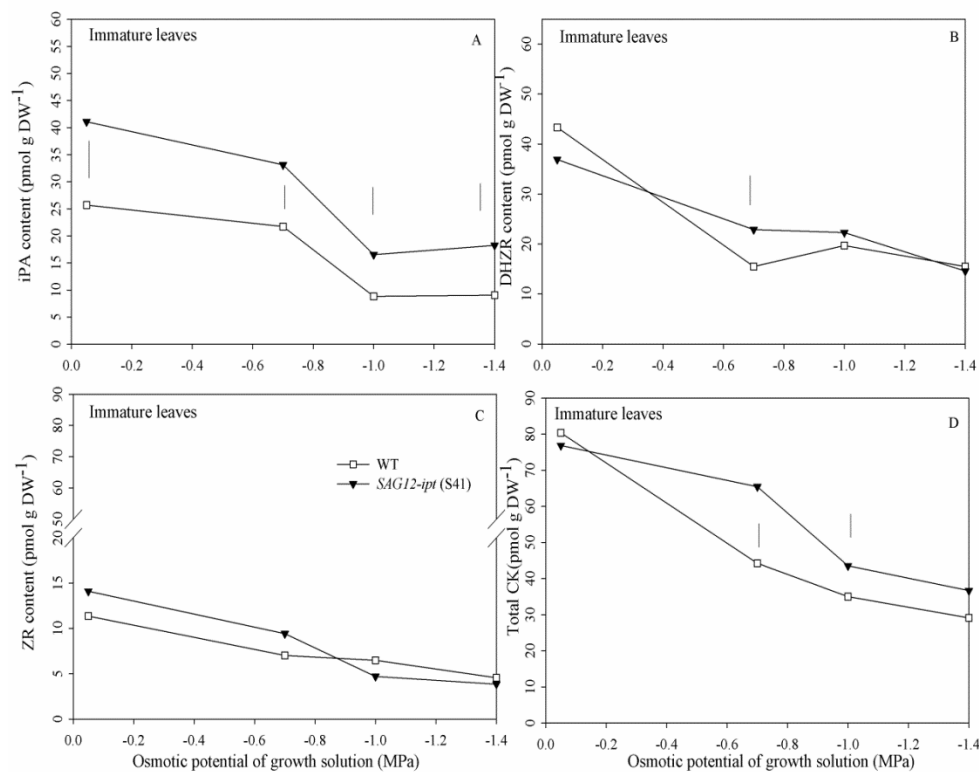
Figure 3. (A) Isopentyladenine (iPa) (B) dihydrozeatin riboside (DHZR), (C) zeatin riboside (ZR) and (D) total cytokinin content (CK) (sum of iPa, DHZR, ZR) of mature (fully expanded) leaves of *SAG12-ipt* creeping bentgrass (line S41) and wild type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (0 to -1.4 MPa). Vertical bars indicate LSD values where significant differences were detected ($P \leq 0.05$) for comparison between plant lines at a given osmotic potential of the growth solution.
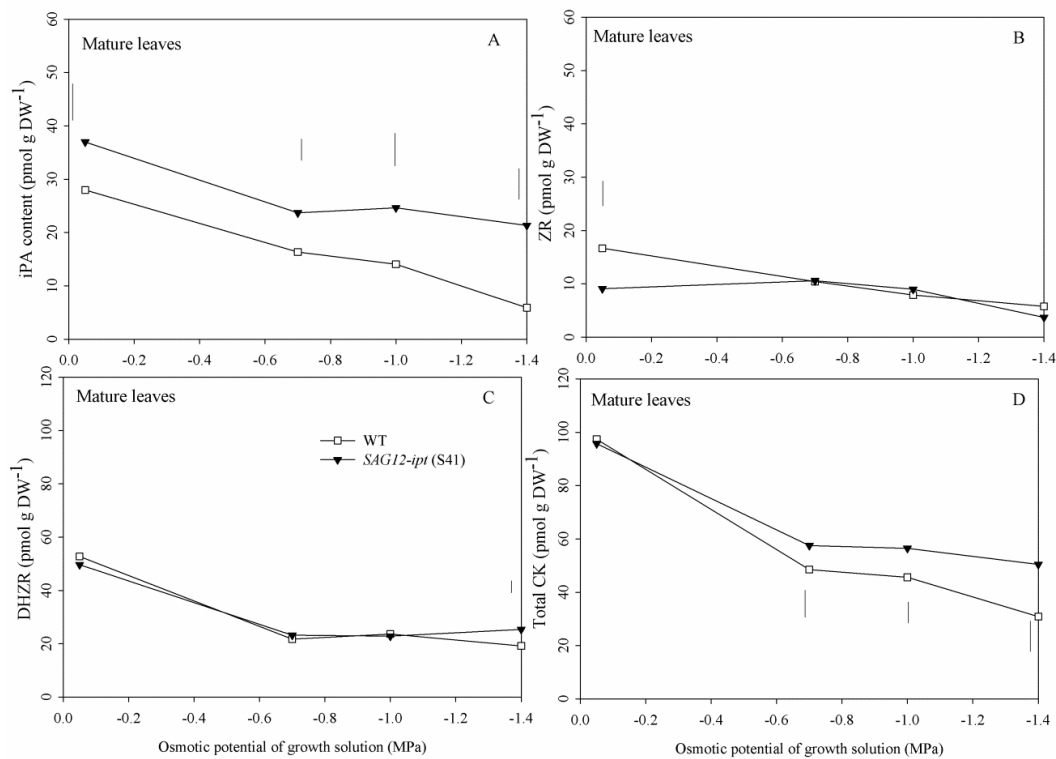
Figure 4. Relative water content (RWC, %) and osmotic adjustment (OA), calculated as the difference in osmotic potential at between fully rehydrated control and PEG-induced water stress treated leaves, of immature (top two leaves not fully expanded) and mature (fully expanded) leaves of *SAG12-ipt* creeping bentgrass (line S41) and wild type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (0 to -1.4 MPa). Vertical bars indicate LSD values where significant differences were detected (*P* ≤ 0.05) for comparison between plant lines at a given osmotic potential of the growth solution.
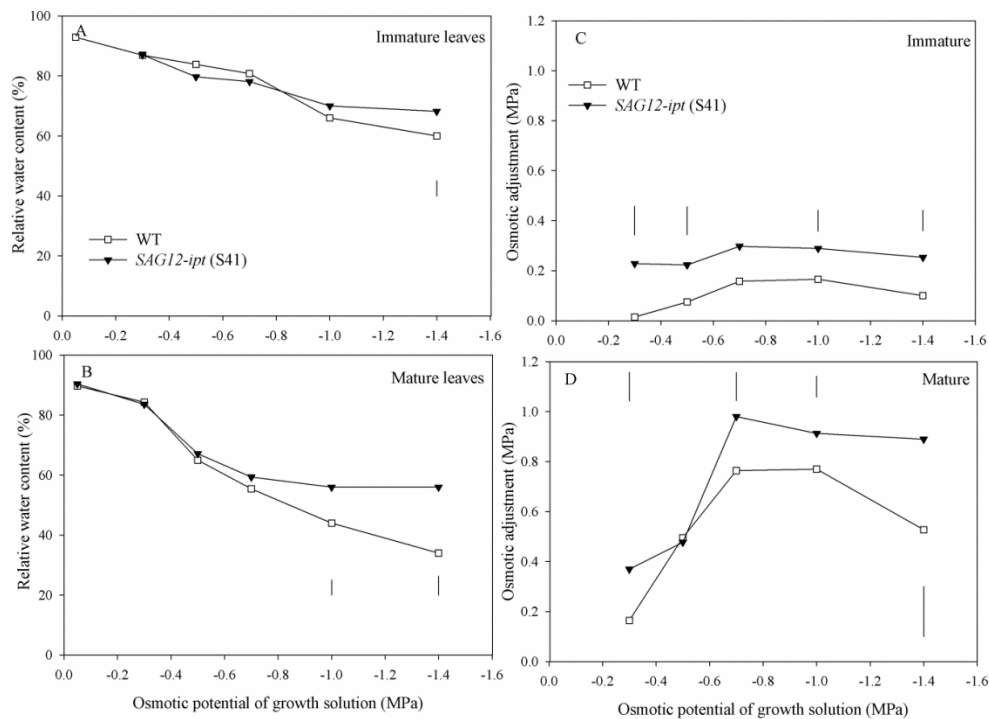
Figure 5. Chlorophyll content, photochemical efficiency, and carbon isotope discrimination of control and PEG-induced water stress treated leaves, of immature (top two leaves not fully expanded) and mature (fully expanded) leaves of *SAG12-ipt* creeping bentgrass (line S41) and wild type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (0 to -1.4 MPa). Vertical bars indicate LSD values where significant differences were detected ($P \leq 0.05$) for comparison between plant lines at a given osmotic potential of the growth solution.
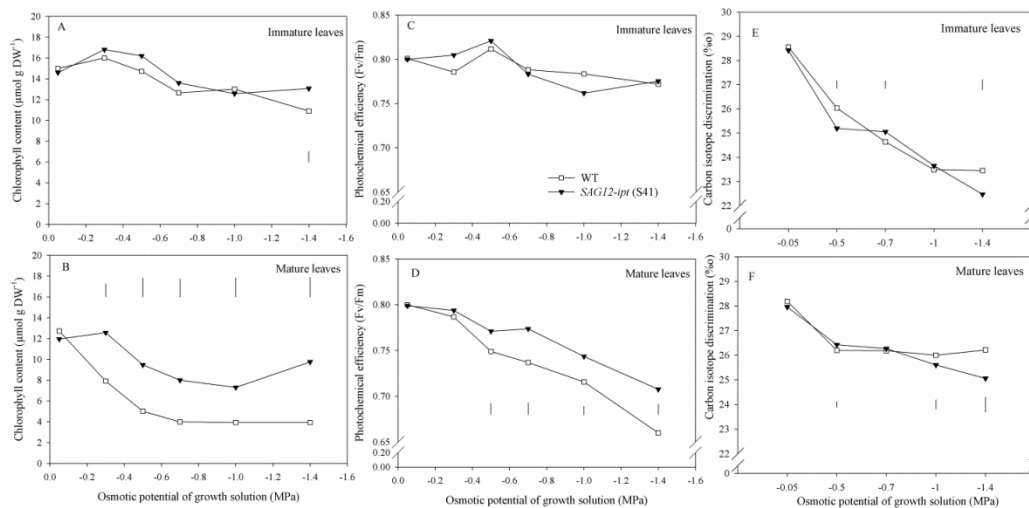
Figure 6. (A) Isopentyl adenine (iPa) (B) dihydrozeatin riboside (DHZR), (C) zeatin riboside (ZR) and (D) total cytokinin content (CK) (sum of iPa, DHZR, ZR) of roots of *SAG12-ipt* creeping bentgrass (line S41) and wild type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (0 to -1.4 MPa). Vertical bars indicate LSD values where significant differences were detected ($P \leq 0.05$) for comparison between plant lines at a given osmotic potential of the growth solution.
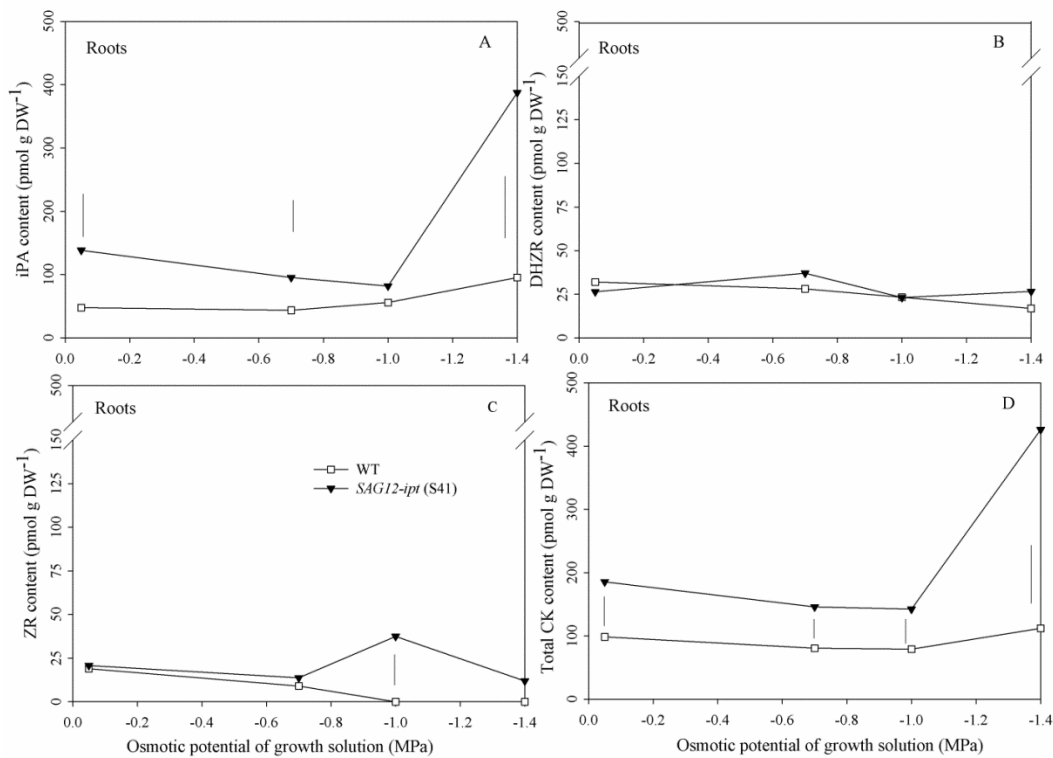
Figure 7. Root viability, as measured by the triphenyl tetrazolium chloride (TTC) reduction method and the absorbance (A) at 490 nm, of *SAG12-ipt* creeping bentgrass (line S41) and wild type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (0 to -1.4 MPa). Vertical bars indicate LSD values where significant differences were detected (*P* ≤ 0.05) for comparison between plant lines at a given osmotic potential of the growth solution.
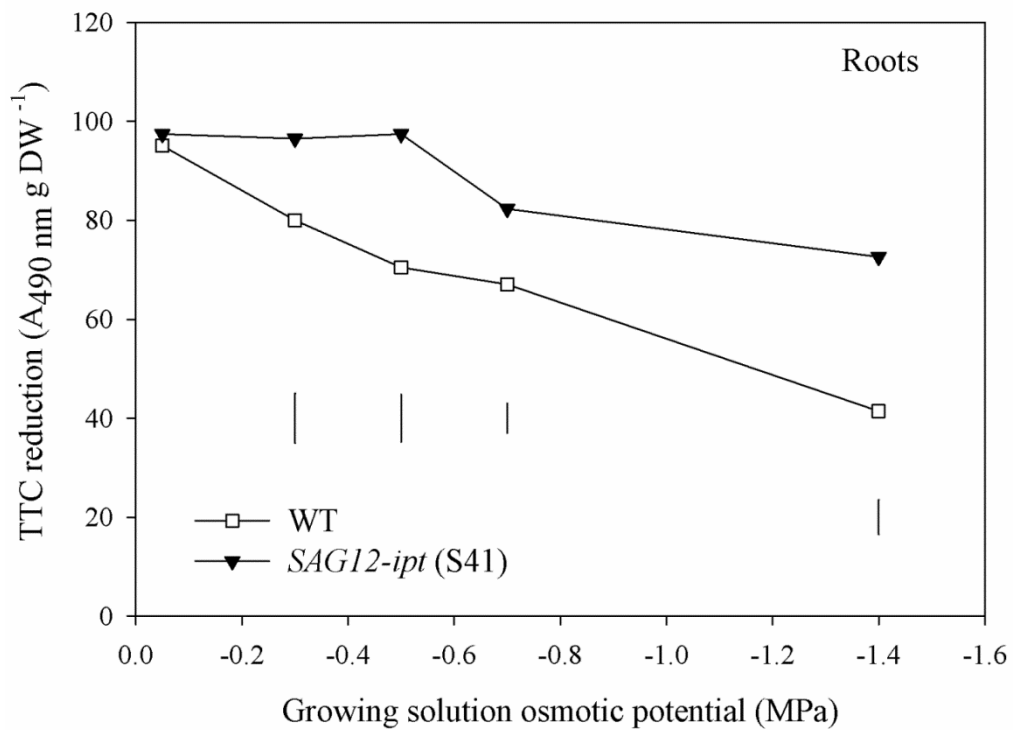
CHAPTER 3

PROTEIN ACCUMULATION IN LEAVES AND ROOTS ASSOCIATED WITH IMPROVED DROUGHT TOLERANCE IN CREEPING BENTGRASS EXPRESSING AN IPT GENE FOR CYTOKININ SYNTHESIS

INTRODUCTION

Exposure of plants to water stress causes many physiological changes within plant cells, including hormonal metabolism and proteomic changes (Salekdeh *et al.*, 2002*a, b*; Davies, 2010). Endogenous cytokinin (CK) biosynthesis, content, translocation, and activity decline in response to water stress (Yang *et al.*, 2002; Kudoyarova *et al.*, 2006). Maintenance of CKs, whether through increasing biosynthesis, reducing CK degradation, or increasing CK stability during stress conditions, has been found to be an important factor regulating plant responses to environmental stress. This has been supported by research using transgenic modification of the CK content in various plant species, such as *Arabidopsis* (*Arabidopsis thaliana*) (Medford *et al.*, 1989; Zhang *et al.*, 2000), lettuce (*Lactuca sativa*) (McCabe *et al.*, 2001), tobacco (*Nicotiana tabacum*) (Rivero *et al.*, 2007, 2009), petunia (*Petunia*×*hybrida*) (Clark *et al.* 2004), tall fescue (*Festuca arundinacea*) (Hu *et al.*, 2005), and creeping bentgrass (*Agrostis stolonifera*) (Xu *et al.*, 2009; Merewitz *et al.*, 2010, 2011).

Transgenic modification of plants to incorporate the *ipt* gene encoding an enzyme in the CK biosynthesis pathway, adenine isopentenyl transferase, increases endogenous CK content, resulting in improved drought tolerance in various plant species (Clark *et al.* 2004; Rivero *et al.*, 2007; Merewitz *et al.*, 2010, 2011; P Zhang *et al.*, 2010). Merewitz *et al.* (2010, 2011) reported that compared with null transformant (NT) control plants, creeping bentgrass (a C_3 perennial grass species) containing the *ipt* gene under a senescence-activated promoter

(*SAG12-ipt*) exhibited higher photosynthesis rates, photochemical efficiency (F_v/F_m), leaf chlorophyll content, osmotic adjustment, and water use efficiency (WUE), as well as enhanced root growth, and root viability under drought stress. Rivero *et al.* (2007) found that *ipt* transgenic tobacco had improved drought tolerance, which was manifested by maintaining a higher water content and photosynthetic activity, and displayed minimal yield loss during drought. They attributed the improved drought tolerance in *SAG12-ipt* transgenic tobacco to the up-regulation of photorespiration protection of photosynthesis under drought stress (Rivero *et al.*, 2009). Clark *et al.* (2004) found that *ipt* transgenic lines of petunia exhibited delayed leaf senescence and increased the number of branches, but decreased adventitious rooting. Transgenic cassava (*Manihot esculenta*) plants with *ipt* maintained higher chlorophyll content and an early storage root bulking in comparison with wild-type plants (P Zhang *et al.*, 2010).

Despite knowledge of CK-mediated drought responses in some monocot species and many dicot species, how *ipt* gene-regulated CK synthesis during drought stress regulates metabolic processes, such as photosynthesis, antioxidant metabolism, osmotic adjustment, and other physiological characteristics underlying drought tolerance, is not well understood. It is commonly known that hormonal and proteomic changes are tightly linked and may coordinately regulate plant responses to drought for stress perception, signalling, and metabolic regulation (Bray, 1997). Questions still remain regarding what specific protein changes may occur in leaves and roots of creeping bentgrass with elevated CK content that has been found to promote drought tolerance. Two-dimensional

PAGE has been widely used to differentiate proteomic responses between drought-tolerant and drought-sensitive plants (Riccardi *et al.*, 1998; C Xu *et al.*, 2008, 2010; Y Xu *et al.*, 2009; Xu and Huang, 2010; Zhao *et al.*, 2011) and has allowed for the successful identification of proteins regulating the plant defence response or cellular damage caused by drought (Riccardi *et al.*, 1998). Identification of specific changes in enzymatic activities and abundance of proteins due to elevated CK content may aid in elucidating the relationship of CKs to various drought protection responses.

The objective of this study was to identify protein changes in both leaves and roots of *ipt* transgenic and NT control plants at the same level of cellular water deficit in order to elucidate mechanisticly how *SAG-ipt* gene-induced elevated CK content contributes to improved physiological drought tolerance in creeping bentgrass.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Transgenic creeping bentgrass plants were produced by the *Agrobacterium* transformation method as described previously (Xu *et al.*, 2009; Merewitz *et al.*, 2010, 2011; Xing *et al.*, 2010). Plant material included a null transformed line of 'Penncross' (NT) and a *SAG12-ipt* transgenic line (S41). The *SAG12* promoter is expressed in an autoregulated manner under stress conditions to prevent excess CKs from accumulating. *SAG12-ipt* expression caused greater levels of CKs and better drought tolerance than in NT plants in previous drought studies (Merewitz *et al.*, 2010, 2011). Transgene expression determined by northern blot analysis and changes in hormone content of *SAG12-ipt* lines under drought stress relative to NT have been previously reported (Merewitz *et al.*, 2010). All plant material was vegetatively propagated in a greenhouse in January 2009 and transplanted into PVC tubes (40 cm in height×10.16 cm in diameter) containing an equal volume of 1:1 fine sand:soil mix (fine-loamy, mixed mesic Typic Hapludult type soil). Greenhouse conditions were controlled to maintain natural light and supplemental sodium lamps when necessary at ∼600 μmol m^{-2} s^{-1} photosynthetic photon flux density at canopy height for a 12 h photoperiod and an average air temperature of 21 °C/14 °C (day/night). Plants were watered daily and fertilized once per week with Hoagland's nutrient solution (Hoagland and Arnon, 1950). Plants were allowed to establish fully in grass canopy and root systems during December–February 2009 for ∼60 d in the greenhouse. Plants were then

transferred to a controlled-environment growth chamber in February 2009

(Conviron, Winnipeg, Canada) where they were acclimated to the growth

chamber conditions for 10 d. The chamber was set to maintain 20/15 °C

(day/night) temperatures, 12 h photoperiod, 60% relative humidity, and 500 µmol

$m^{-2} s^{-1}$ photosynthetic photon flux density at canopy height. Watering treatments

were imposed in the growth chamber on 3 March 2009.

Water Stress Treatments

Water treatments consisted of a well-watered control or water stress by

withholding irrigation for both NT and *SAG12-ipt* plants (40 plants of each). Soil

volumetric water content (SWC) was determined with the time domain

reflectometry (TDR) method (Topp *et al.*, 1980) using a Trase TDR instrument

(Soil Moisture Equipment Corp., Santa Barbara, CA, USA). SWC was measured

with three-pronged waveguide probes (20 cm in length, spaced 2.54 cm apart)

installed vertically in each pot, four probes in the control treatment and four

probes in the water stress treatment (four replicates in each line). Pot capacity of

the soil water was ~25%.

Physiological Evaluation

All physiological measurements and protein sampling were carried out on

four replicated pots when the SWC reached an average of 22, 18, 15, 10, or 5% in

pots of both plant lines, which occurred over a period of 14 d of water stress

treatment. This was done so that comparisons within the physiological attributes

and protein responses between lines can be made at a given soil moisture level or at the same water deficit level.

Grass quality was visually rated based on leaf colour and density on a scale of 1–9, with 1 as a completely brown and desiccated canopy, 6 as the minimal acceptable level, and 9 as a turgid, green, and dense canopy (Turgeon, 2008). Relative water content (RWC) of leaves was measured to determine the leaf hydration status for comparison of protein changes at a given level of leaf RWC. Leaf RWC was calculated based on fresh weight (FW), turgid weight (TW), and dry weight (DW) of ~0.1 g of leaf samples. Leaf FW was determined on a mass balance immediately after being excised from the plants. TWs were determined after soaking the leaves in de-ionized water for 12 h in a covered Petri dish; they weighed immediately after they had been blotted dry. Leaves were then dried in an 80 °C oven for at least 72 h prior to being weighed for DW. RWC was calculated using the formula: (FW−DW)/(TW−DW)×100 (Barrs and Weatherley, 1962).

Leaf electrolyte leakage (EL) measurement was performed to estimate cell membrane stability and indicate drought damage severity. Leaf samples of ~10 leaves were taken from each plant, washed in de-ionized water four times, immersed in 25–30 ml of de-ionized water, and placed on the shaker for 24 h. The conductivity of the immersion water containing the living leaf tissue was measured as initial conductivity (C_i). The samples were then autoclaved, placed on the shaker for 24 h, and the conductivity of the resulting water containing the

dead tissue was measured as maximum conductivity (C_{max}). The percentage EL was calculated as $C_i/C_{max}\times100$ (Blum, 1981).

Leaf photochemical efficiency (F_v/F_m) was evaluated as the ratio of the variable fluorescence (F_v) to the maximal fluorescence (F_m) value determined using a chlorophyll fluorescence meter (Fim 1500; Dynamax, Houston, TX, USA). Leaf clips were used to adapt individual leaves to darkness for 30 min prior to reading the F_v/F_m ratio with the fluorescence meter. Two subsamples were taken per pot on each sampling day.

Roots were harvested by destructive sampling of individual plants at a given level of SWC. Roots were shaken free of soil over a sieve, quickly rinsed, and patted dry to minimize exposure to water during sampling. Roots were immediately frozen in liquid N until further analysis. The root to shoot ratio was calculated as root DW:shoot DW of the sum of all roots and shoots collected from each individual plant after washing the roots free of soil. Roots and leaves were dried in an oven at 80 °C for at least 72 h prior to being weighed for DW.

Antioxidant Activity and Malondialdehyde (MDA) Content

Activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), and MDA content were determined based on the protocols described in Xu and Huang (2004). Briefly, a fresh leaf or root sample of ∼0.5–1.0 g was collected from each plant, frozen immediately in liquid nitrogen, and stored at − 80 °C until use. For enzymes and MDA extraction, frozen samples were

homogenized with 7 ml of 50 mM phosphate buffer solution (pH 7.0), ground in a

mortar on ice, and centrifuged at 20 000 g for 25 min at 4 °C. The supernatant was

used to evaluate total soluble protein, enzyme activity, and MDA content. Protein

content was based on comparison with bovine serum albumin (BSA) as a standard

(Bradford, 1976). SOD activity was measured according to the method of Zhang

and Kirkham (1996) and Xu and Huang (2004). One unit of SOD activity was

defined as the amount of SOD required to cause 50% inhibition of nitroblue

tetrazolium (NBT) reduction at 560 nm min^{-1}. CAT and POD activity were

determined based on the method of Chance and Maehly (1955) as described in

detail for creeping bentgrass in Xu and Huang (2004). Enzyme activities were

based on the absorbance change of the reaction solution per minute at a given

wavelength for each enzyme: CAT at 240 nm and POD at 470 nm. MDA content

was measured at 532 nm and 600 nm after reaction of the extraction solution with

trichloroacetic acid and thiobarbituric acid using the method of Dhindsa *et al.*

(1981). The formula used for calculation of MDA content was A_{600} subtracted

from A_{532} multiplied by the extinction coefficient of 155 mm^{-1} cm^{-1} for MDA

(Heath and Packer, 1968). All reaction solutions, non-reacted control solutions,

and standards were analysed at a given wavelength with a spectrophotometer

(Spectronic Instruments, Inc., New York, NY, USA). Protein content for the

activity assays was determined using the method of Bradford (1976). A 10 μl

aliquot of each protein extract was mixed with 0.5 ml of dye reagent (diluted five

times) (Bio-Rad Laboratories, Hercules, CA, USA). The absorbance values of

each extract were measured in a spectrophotometer at 595 nm at regular intervals

for 30 min. The obtained curves were compared with a standard curve developed by treating a known amount of BSA in the same fashion.

Protein Extraction and Quantification

Leaf and root samples (a mixture of immature and mature tissues) were harvested separately from each pot on a given sampling day as determined by the SWC. The samples were immediately placed in liquid nitrogen and stored at −80 °C until further analysis. A known mass of leaves and roots was ground to a fine powder with liquid nitrogen using a pestle and mortar and used for subsequent analysis. Total proteins were extracted using the trichloroacetic acid/acetone method described by Xu *et al.* (2008). About 0.5 g of leaf or 1 g of root samples were homogenized on ice in 10 ml of precipitation solution (10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone) for 10 min and incubated at –20 °C for 2 h. The protein pellet was collected and washed with cold acetone containing 0.07% 2-mercaptoethanol until the supernatant became colourless. Pellets were then vacuum-dried, suspended in re-solubilization solution [8 M urea, 2 M thiourea, 2% CHAPS, 1% dithiothreitol (DTT), and 1% pharmalyte], and then centrifuged at 21 000 g for 20 min. The supernatant containing the proteins was saved for quantification after being stored at –20 °C.

Two-dimensional PAGE and Image Analysis

Protein extract samples from well-watered plants and from the 47% RWC drought stress level were run in the first dimension isoelectric focusing (IEF) by

using an IPGPhor apparatus (GE Healthcare, Waukesha, WI, USA) as described

in detail in Xu *et al.* (2008). Briefly, each sample contained 300 μg of protein and

was subjected to IEF in immobilized pH gradient strips (pH 3.0–10.0, linear

gradient, 13 cm). Following IEF, the strips were equilibrated twice for 15 min at

room temperature in 50 mM TRIS-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2%

(w/v) SDS, 0.002% (w/v) bromophenol blue, and 1% (w/v) DTT and then

incubated with the same buffer containing 4.0% (w/v) iodoacetamide instead of

DTT for 20 min. Gel electrophoresis for the second dimension was run in an SE

600 Ruby electrophoresis apparatus (GE Healthcare, Waukesha, WI, USA) in a

12.5% SDS–polyacrylamide gel. The running conditions were 5 mA per strip for

30 min followed by 20 mA per strip for 5 h. The gels were stained with

Coomassie brilliant blue G-250 and scanned using a Personal Densitometer SI

(63-0016-46, GE Healthcare). Gel images were scanned for relative protein

content using Progenesis SameSpots software (Nonlinear Dynamics, Durham,

NC, USA) with automatic default spot analysis settings including normalization

with the total percentage volume of all spots on the gel to correct for potential

variation due to staining. Manual correction and editing of spots where

appropriate was also performed and were included in the analysis.

Protein Identification and Categorization

Selected protein spots were manually excised from gels and subjected to

trypsin digestion. The resulting peptides were analysed by matrix-assisted laser

desorption/ionization (MALDI) or liquid chromatography-quadrupole (LCQ)

followed by time-of-flight mass spectrometry (TOF-MS) as described by Xu *et al.*
(2008). Data were searched against the National Center for Biotechnology
Information (NCBI) database and a protein identification database called the
MASCOT search engine (V1.9, Matrix Science, Boston, MA, USA) on a group-
based phosphorylation scoring (GPS) server (V. 3.5, Applied Biosystems,
Framingham, MA, USA). Proteins containing at least two peptides with a
confidence interval >95% were considered accurately identified. The obtained
sequence was also manually assigned to perform another search in the Swiss-Prot
and TrEMBL databases (Universal Protein Resource, UniProt Consortium, 2011)
using a text format known as FASTA (Lipman and Pearson, 1985). Proteins were
categorized by their function based on the system used previously in Bevan *et al.*
(1998) and C Xu *et al.* (2010). Proteins that were differentially expressed but not
picked for identification are labelled as unknown followed by the spot number
(u#). Protein spots aligning with those previously identified in Xu *et al.* (2008,
2009) are labelled with the same spot identification numbers (SIDs) that were
previously reported. Spots reported previously in Y Xu *et al.* (2010) are labelled
with a Y followed by a number (Y#). Protein spots not previously identified that
were picked for identification in this study are labelled as L followed by the spot
number (L#) for leaves and R followed by a number (R#) for roots.

Experimental Design and Statistical Analysis

The experimental design was a split-plot design with irrigation treatment
as the main plots and plant materials as the subplots, with four replicates for each

irrigation treatment, destructive sampling day at a given SWC, and grass type

(totalling 40 plants of each plant type). The effects of watering treatment, plant

materials, and corresponding interactions were determined by analysis of variance

according to the general linear model procedure of SAS (version 9.0; SAS

Institute, Cary, NC, USA). Differences between watering treatments and plant

means were separated by Fisher's protected least significance difference (LSD)

test at the 0.05 probability level.

RESULTS

Soil Water Status Indicating the Level of Soil Water Stress

SWC was maintained at ~20–25% for well-watered plants for both NT and *SAG12-ipt* plants (Fig. 1). The SWC for both the NT and *SAG12-ipt* plants declined gradually after irrigation was withheld. It dropped to 5% after 11 d of water stress, and did not differ between pots of the NT and *SAG12-ipt* plants, indicating that all plants were exposed to the same level of water deficit (Fig. 1).

Leaf Physiological Responses to Water Stress

Grass quality, EL, RWC, and F_v/F_m were not significantly different between the NT and *SAG12-ipt* plants under well-watered conditions or at the initiation of water stress treatment when SWC was maintained at 25% (Fig. 2). Leaf colour and turgidity estimated as grass quality of both NT and *SAG12-ipt* decreased in response to water stress, but at a slower rate of decline, and were maintained at a significantly higher level for *SAG12-ipt* plants than the NT plants (Fig. 2A). At the end of the water stress treatment when the SWC declined to 5%, quality ratings were maintained at 4 for *SAG12-ipt* and only 1 for NT plants. Leaf EL, RWC, and F_v/F_m also declined in response to water stress in both the NT and *SAG12-ipt* plants (Fig. 2C, D). Leaf F_v/F_m declined when SWC dropped to 5% and was significantly higher in *SAG12-ipt* (0.7) than in NT plants (0.5). The decline of RWC and the corresponding increase in EL occurred at a higher SWC

for NT relative to *SAG12-ipt* plants. Leaf RWC dropped to ~7% when SWC

reached 10% for NT and 5% for *SAG12-ipt*.

Leaf Antioxidant Enzyme Activity and Lipid Peroxidation

Under well-watered conditions at 25% SWC and throughout water stress

treatments (15–5% SWC), leaf SOD, CAT, and POD had significantly higher

activity in *SAG12-ipt* plants compared with NT plants (Fig. 3). SOD and POD

activities were relatively unresponsive to decreasing SWC from 25% to 5% in

both NT and *SAG12-ipt* leaves. CAT activity was relatively unchanged during

water stress in NT leaves, but increased ~2-fold in *SAG12-ipt* leaves exposed to

5% SWC compared with that at 25% SWC. Lipid peroxidation estimated by

MDA content increased during water stress, particularly for the NT plants. Leaf

MDA content was 51% greater in NT compared with *SAG12-ipt* plants at 47%

RWC and was greater at all levels of water stress from 15% to 5% SWC (Fig.

3D).

Root Physiological Responses to Drought Stress

Root:shoot ratios increased with decreasing SWC in both NT and *SAG12-*

ipt plants, but the ratios were significantly greater in the *SAG12-ipt* plants at 10%

and 5% SWC (Fig. 4A). Root MDA content increased with declining SWC from

25% to 5% in NT plants, but the increases in root MDA content did not occur

until SWC decreased to 5% in *SAG12-ipt* plants (Fig. 4B). At an SWC between

25% and 10%, the NT roots had significantly higher MDA content than those of

the *SAG12-ipt* plants. Root SOD activity decreased during the decline in SWC

from 25% to 5% in both NT and *SAG12-ipt* plants, but the *SAG12-ipt* plants had

significantly greater SOD activity in roots than the NT roots at 25, 10, and 5%

SWC (Fig. 4C). Root POD activity was relatively unchanged by decreasing SWC

and was not significantly different between *SAG12-ipt* and NT roots at an SWC

between 25% and 10%. At 5% SWC, POD activity was significantly higher in NT

roots. No difference in CAT activity was detectable in roots of both NT and

SAG12-ipt plants (data not shown).

Proteins Exhibited Differential Responses to SAG12-ipt Expression and Water Stress

A total of 431 protein spots were detected in each leaf sample and 315

spots were detected in each root sample. Representative gel images depicting

protein spot numbers are shown in Fig. 5. A total of 64 protein spots from leaves

and 83 spots from roots remain unidentified due to technical reasons such as

insufficient quantity in the gel for identification. These spots are labelled with 'u'

followed by a number in the gel images and will not be discussed further. The

specific proteins in leaves (Table 1) or roots (Table 2) either responsive to water

stress (decreased or increased abundance compared with the well-watered control

columns 2 and 3) or altered by the transgene expression (different abundance

levels from the water-stressed NT plants in column 4) were identified and were

placed into the following categories: metabolism, energy, cell growth/division,

protein synthesis, protein destination/storage, cell structure, signal transduction,

disease/stress defence, secondary metabolism, and unclear (unknown function or unsuccessful identification).

For both leaves and roots, the total number of proteins that exhibited either an increase or a decrease in abundance relative to their respective control line are displayed in Fig. 6 and are shown as a percentage within each category in Fig. 7. In response to water stress, more proteins exhibited a decrease in abundance than an increase in abundance in both plant lines. Among other differences, the total protein number that decreased only in NT leaves was greater than those that decreased only in *SAG12-ipt* leaves and roots. Of particular interest may be the six proteins in leaves and the four proteins in roots that increased in *SAG12-ipt* but decreased in NT (Fig. 6). In both leaves and roots, the changes primarily occurred in proteins related to energy and metabolism (Fig. 7). A greater percentage of proteins in *SAG12-ipt* leaves increased in the energy category than in NT plants (Fig. 7A). For roots, secondary metabolism decreased in both plant lines, but more in NT plants (Fig. 7B). Specific protein changes will be discussed in greater detail below.

Specific Proteins Responsive to *SAG12-ipt* Expression under Non-stress Conditions

Protein changes due to the presence of the transgene were determined by comparing proteins present in the two- dimensional gels derived from the well-watered control of NT with those of *SAG12-ipt* plants. In leaves, the abundance of 12 proteins was significantly higher in *SAG12-ipt* plants compared with NT, and

11 spots were identified (Table 1, column 1). These included seven proteins in the energy category, chloroplastic and cytosolic forms of glyceraldehyde phosphate dehydrogenase (GAPDH; leaf 11, 49, L36), two isoforms of the ribulose 1,5-bisphosphate carboxylase (RuBisCO) small subunit (leaf 29, 30), photosystem I subunit (PSI subunit K; PSAK) (leaf 88), and a putative phosphogluconate dehydrogenase (6PGDH; leaf L31); one protein in the protein destination/storage category [OSJNBa0039C07.4 (L34)]; two proteins involved in stress defence [CAT isoforms (leaf 111, L23)], and one with unknown function (leaf L32). The abundance of five proteins was lower in *SAG12-ipt* leaves relative to NT leaves under well-watered conditions (Table 1, column 1), of which four were identified. They were all in the energy category, including a RuBisCO small subunit (leaf 28), a chloroplastic aldolase (leaf 63), and the ATPase β-subunit (leaf 76, 77).

In roots, the abundance of 10 proteins was higher in *SAG12-ipt* plants relative to the NT line under well-watered conditions (Table 2, column 1). Of these protein spots, eight were identified, including one in metabolism (a nucleotide-sugar dehydratase), four in energy [two forms of GAPDH (root 53/R13, R14), and two forms of isocitrate dehydrogenase (IDH; root 78, 79)], one in protein synthesis (a putative asparagine-tRNA ligase, root 57), one in secondary metabolism (UDP-glucose 6- dehydrogenase, root 68), and one unknown (root R16). The abundance of five proteins was significantly lower in *SAG12-ipt* relative to NT, and three of these were identified. These included one in protein destination/storage [a protein disulphide isomerase 3 (PDI3) precursor,

root 90], one in energy (a ferredoxin-nitrite reductase precursor, root R44), and
one unknown (root R16).

Specific Proteins Responsive to *SAG12-ipt* Expression under Water Stress

When compared as a percentage of the control, the abundance of 12
proteins increased and of 39 decreased in leaves of water-stressed NT plants (Fig.
5 and Table 1, column 2). In *SAG12-ipt* leaves, 16 protein spots exhibited
increased abundance and 23 had decreased abundance under water stress (Fig. 5,
and Table 1, column 3). Out of these water stress-responsive proteins for both
SAG12-ipt and NT (Table 1, columns 2 and 3), 37 proteins exhibited a similar
trend in change in response to water stress, either decreased or increased in
abundance in both the NT and transgenic plants, whereas 69 proteins exhibited a
differential responses to water stress between the NT and *SAG12-ipt* plants
(unchanged, increased, or decreased in either the NT or *SAG12-ipt* plants or
decreased/increased in the NT versus *SAG12-ipt* plants). When comparing plant
lines under water stress (Table 1, column 4), 26 proteins had greater abundance
and 14 had lower abundance in *SAG12-ipt* plants than those in the NT plants.

In the roots, the abundance of 25 protein spots increased under water
stress and that of 28 proteins decreased relative to the control condition in NT
plants (Fig. 5 and Table 2, column 2). In *SAG12-ipt* roots, the abundance of 22
protein spots increased and of 13 decreased under water stress (Fig. 5, and Table
2, column 3). Comparing root protein changes between *SAG12-ipt* and NT plants

(comparing column 2 with 3, Table 2), 29 proteins had the same trend in

accumulation in response to water stress (significantly greater or lower

accumulation) whereas 54 proteins had differential accumulation in response to

water stress (either unchanged, increased, or decreased in one line, but not the

other, or with the opposite trend) in NT and *SAG12-ipt* roots. When comparing

both plant lines under water stress (Table 2, column 4), 13 proteins had greater

and 17 proteins had lower abundance in *SAG12-ipt* relative to NT plants.

DISCUSSION

Physiological Characterization of Improved Drought Tolerance in

***SAG12-ipt* Transgenic Plants**

Previous studies demonstrated that expressing *SAG12-ipt* during drought treatment enhanced drought tolerance in creeping bentgrass and was associated with increases in shoot and root growth, photosynthetic activities, and WUE compared with NT plants exposed to drought stress (Merewitz *et al.*, 2010, 2011). In the current study, the analysis of physiological responses to water stress for the NT and *SAG12-ipt* plants demonstrated that *ipt* expression in creeping bentgrass could alleviate water stress damage to cellular membranes and photochemical systems for photosynthesis, as manifested by lower EL and MDA content and higher F_v/F_m in *SAG12-ipt* plants, which helped maintain greater cellular hydration (RWC) and grass quality under water stress.

The water depletion rate, as indicated by changes in SWC content (Fig. 1) during water stress, was similar between the NT and *SAG12-ipt* plants, but at the same level of SWC and RWC (i.e. 47% RWC), transgenic plants maintained lower EL and MDA content. These data suggested that the improved shoot and root growth in the *SAG12-ipt* plants under water stress was not related to avoidance mechanisms such as a differential water depletion rate, but rather could be due to enhanced tolerance mechanisms, which is supported by the analysis of

antioxidant enzyme activities. Leaf SOD, CAT, and POD activity and root SOD

activity were significantly higher in *SAG12-ipt* plants compared with those in the

NT plants. The tolerance mechanism most directly affected by the *ipt* gene may

be increased antioxidant activity, rather than the direct regulation of water loss

and cellular dehydration.

Proteomic Changes Associated with *SAG12-ipt* Expression and Water Stress

The expression of *ipt* in creeping bentgrass caused changes in protein

abundance under both well-watered and water stress conditions. The differential

protein expression in *SAG12-ipt* compared with NT in well-watered plants was

presumably due to *ipt* expression associated with natural leaf senescence. PCR-

based transcript expression of *SAG12-ipt* was detected under non-stressed

conditions previously in creeping bentgrass (Merewitz *et al.*, 2011), and similar

findings have been reported in petunia (*Petunia×hybrida*) (Clarke, *et al.*, 2004),

maize (*Zea mays*) (Robson *et al.*, 2004), and tobacco (Rivero *et al.*, 2007). The *ipt*

expression under non-stress conditions was associated with higher levels of

isopentenyl adenine (iPa) in immature leaves, mature leaves, and roots, and higher

zeatin riboside (ZR) in mature leaves of creeping bentgrass (Merewitz *et al.*,

2011). The differential protein accumulation in non-stressed NT and *SAG12-ipt*

plants, as described in the Results above, is due to the elevated CKs and

expression of *ipt* associated with natural leaf or root senescence.

Since the major objective of this study was to identify proteins altered due to *SAG12-ipt* expression that may contribute to improved drought tolerance, the following discussion is focused on proteins with known important functions that exhibited differential responses to water stress due to the *SAG12-ipt* gene in creeping bentgrass. These proteins are discussed below in terms of their biological functions related to drought tolerance. The analysis of *SAG12-ipt*-regulated protein changes under water stress may reveal the identity of important metabolic pathways contributing to increased drought tolerance as demonstrated in previous studies (Merewitz *et al.*, 2010, 2011), and from the physiological results discussed above.

Protein Changes within the Metabolism Category

Differential responses in the metabolic enzymes in both leaves and roots between the NT and transgenic plants were primarily related to enzymes involved in amino acid and cell wall degradation or biosynthesis. Changes in amino acid content have downstream effects on protein synthesis and other stress responses such as osmotic adjustment (OA). Cell wall-modifying enzymes may affect cell wall elasticity, thereby regulating cell turgor (Bohnert and Jensen, 1996). The abundance of two proteins related to methionine metabolism, methionine synthase (MS; leaf 9, 10; root 73) and *S*-adenosylmethionine synthetase (SAMS; leaf L27; root 40, 41, R18), was increased and unchanged, respectively, in *SAG12-ipt* in response to water stress, whereas their accumulation decreased in leaves and roots of the NT plants. The activation of MS is an early response to drought symptoms

since increased flux through the pathway provides a source of carbon under stress. During severe stress, MS activity declines. SAMS is downstream of MS and can be a source of methyl groups for key secondary metabolites such as osmoprotectants involved in OA (Bohnert *et al.*, 1996). Thus, the increase or maintenance of MS and SAMS content in *SAG12-ipt* plants may reflect more active methionine and osmoregulant metabolism than in the NT plants under water stress. Similarly, induction of MS transcripts and an increase in MS protein content under salt stress has been associated with salt stress tolerance in barley (*Hordeum vulgare*) (Narita *et al.*, 2004).

Other proteins in the metabolism category that were differentially accumulated within the plant lines, such as aspartate and alanine aminotransferases, also may be involved in OA as well as in the activation of antioxidant enzymes to reduce the amount of reactive oxygen species (ROS) generated by drought stress (Kocsy *et al.*, 2005). Increased levels of aspartate aminotransferase in *SAG12-ipt* plants (leaf 11) may allow for increased OA under water stress conditions, but, since the other isoform of this enzyme (leaf L24) was reduced by water stress in *SAG12-ipt*, the trend in accumulation of this enzyme is unclear. The accumulation of these enzymes is highly dependent on the level of free precursors for aspartate synthesis (Good and Zaplachinski, 1994). In roots, glutamine synthetases (GSs) accumulated more in *SAG12-ipt* than in NT in response to water stress (root 4, 71, 72). Root cytosolic GS is involved in the assimilation of ammonia, N transport/remobilization, and control of root biomass, and GS content/activity typically decreases in response to drought stress (Bauer *et*

al., 1997; Limami *et al.*, 1999). Increased expression of GS genes contributes to drought and salt tolerance (Kalamaki *et al.*, 2009). This is consistent with the increased levels of N-metabolizing enzymes such as IDH reported here and discussed in the energy section. Increased flux through N metabolic pathways suggests an enhancement of N uptake by the roots under stress, which is important for plant stress tolerance.

Some leaf proteins involved in metabolism were at lower levels in *SAG12-ipt* plants compared with NT plants, including isoforms of cell wall β-glucosidase (β-D-glucan exohydrolase) (leaf 12, 13, 14), glycine decarboxylase P subunit/Victorin-binding protein (leaf 3), aminomethyltransferase (leaf 6), and glycolate oxidase (leaf Y110). Cell wall β-glucosidases may have a role in cell wall reinforcement (Ricardi *et al.*, 1998; Dietz *et al.*, 2000; Caruso *et al.*, 2009); however, the role of β-glucosidase in drought stress is not well understood. β-Glucosidases are also implicated in the release of active CKs and abscisic acid (ABA) from inactive forms during stress. Perhaps lower CK content in NT plants and more severe stress led to increased levels of this enzyme in order to release active CK to a greater extent than in *SAG12-ipt* plants. Glycine decarboxylase and aminomethyltransferase are involved in the breakdown of glycine, which when present in the form of glycine betaine is involved in OA during drought stress (Chen and Murata, 2008). Since maintenance of turgor and OA are tightly linked, lower levels of these proteins under water stress in *SAG12-ipt* plants compared with NT plants at the same level of RWC could indicate a reduction in cellular damage in *SAG12-ipt* plants, thereby requiring less cell wall modification and OA

to maintain turgidity. Glycolate oxidase is typically induced by drought stress since it is a key factor in photorespiration and may increase endogenous H_2O_2 (Ingram and Bartels, 1996). Greater levels of this protein in NT plants may also indicate that NT plants were experiencing greater stress damage than *SAG12-ipt* plants.

Protein Changes within the Energy Production Category

In leaves, photosynthetic proteins such as RuBisCO large subunits accumulated to a greater extent in *SAG12-ipt* leaves (leaf 18, 20, 26), whereas these proteins either significantly declined or were unchanged in NT leaves in response to water stress relative to their respective control plants. The greater levels of RuBisCO subunits in leaves in *SAG12-ipt* bentgrass under well-watered and water stress conditions are consistent with results found in *ipt* transgenic tobacco under non-stressed conditions (Rivero *et al.*, 2009) and *SAG12-ipt* creeping bentgrass under heat stress (Y Xu *et al.*, 2010), which showed higher levels of RuBisCO transcripts than the non-transgenic plants. The increase in the abundance of RuBisCo large subunits and the decrease in small subunits have also been found in non-transgenic wheat in response to drought (Caruso *et al.*, 2009). Other proteins involved in photosynthesis that were generally greater in *SAG12-ipt* under water stress than in NT relative to their respective controls were chloroplast precursors (leaf 83, 89, 92, Y172) or those involved in the electron transport chain such as PSI proteins (leaf 88, 91, L18, L7/135), oxygen-evolving complexes (OEEs; spots 80, 83), cytochrome complexes (leaf 92, L2), and a

ferredoxin (leaf L6). A reduction in the rate of chlorophyll degradation in *SAG12-ipt* leaves under water and heat stress (Merewitz *et al.*, 2010, 2011; Y Xu *et al.*, 2010) and the maintenance of F_v/F_m and chloroplast proteins demonstrated in this study are likely to be determinants of sustained CK action under stress, since adequate chloroplast development is necessary for CKs to elicit a growth response in leaves (Kulaeva *et al.*, 2002).

In addition, CKs promote chloroplast development and synthesis of photosynthetic enzymes, and contribute to the maintenance of RuBisCO content and activity under stress conditions (Chernyad'ev, 2009; Davies, 2010). CKs are tightly linked to the acceleration of the biosynthesis of chloroplast electron transport chain proteins such as in PSI and OEEs (Kusnetov *et al.*, 1994). Maintenance of proteins involved in the light reaction of photosynthesis, such as OEEs, is critical for PSII stability under salt stress (Koichi *et al.*, 2000). The abundance of carbonic anhydrase (CA; leaf 96) increased in response to water stress in NT leaves but was not significantly changed in *SAG12-ipt* plants. This enzyme is involved in regulating the concentration of CO_2 within chloroplasts in order to increase the carboxylation rate of RuBisCO. It is possible that the increase in CA found in this study in NT plants could be related to cell damage, since an increase in CA has been documented in response to drought stress damage and elevated levels of ABA (Popova *et al.*, 1996).

Proteins involved in respiration pathways such as glycolysis were responsive to both the *SAG12-ipt* transgene and water stress in both leaves and

roots. The majority of GAPDH isoforms detected were elevated in *SAG12-ipt*

plants in both leaves (leaf 11, 45, 47, 49, 51, 54, 55, 56, 57, L4) and roots (root

R3, 53/R15, R14) under water stress conditions, whereas the abundance of

GAPDH either remained unchanged or decreased in response to water stress in

NT plants. GAPDH catalyses a key step in glycolysis that breaks down glucose

into carbon and energy. The higher levels of the cytosolic form of GAPDH in the

SAG12-ipt plants relative to NT plants under well-watered conditions may reflect

less glycolysis characteristic of natural leaf senescence and may predispose

SAG12-ipt plants to enhanced tolerance. Under stress, it has been found that

GAPDH transcription and protein abundance levels increased in some plant

species (Yang *et al.*, 1993; Chang *et al.*, 2000; Ferreira *et al.*, 2006). GAPDH may

increase in response to stress initially, since it is often an immediate response to

drought stress (Ingram and Bartels, 1996), and then decline as cellular damage

and proteolytic activity increase. Velasco *et al.* (1994) showed that extremely

drought-tolerant resurrection plants exhibited up-regulation of the cytosolic form

of GAPDH transcripts, and a rapid stimulation of glycolysis was an important

characteristic in the drought response to maintain available energy under stress.

Also, recently, GAPDH was found to be a direct target of CK action (Heintz *et*

al., 2006) and is believed to be involved in the stress defence via the antioxidant

defence system by prevention of hydrogen peroxide-mediated cell death (Baek *et*

al., 2008). Promotion of photorespiratory processes was also indicated in *ipt*

transgenic tobacco under water stress (Rivero *et al.*, 2009). Thus, the decline in

GAPDH in NT leaves and the ability of *SAG12-ipt* plants to maintain or increase

GAPDH content and glycolysis could be a significant component contributing to drought tolerance by promoting both energy production and antioxidant defence.

Other proteins involved in respiration, such as leaf and root aldolases (leaf 61, 63, 67; root 52), leaf triose phosphate isomerases (TPIs; leaf 70, L19), and leaf enolase (leaf 79), were generally unchanged or down-regulated by water stress in both *SAG12-ipt* and NT plants. Similar results for these enzymes were found in creeping bentgrass leaves under salt stress (C Xu *et al*, 2010). Interestingly, in roots, IDH content was greater in the non-stressed condition in *SAG12-ipt* than in NT plants. Under drought stress, TPI (root 81) and enolase (root 82, 83) were increased in *SAG12-ipt* but not in NT plants, and root sucrose synthase (root 52) was maintained in *SAG12-ipt* but reduced in NT plants. These differences suggest an activation of glycolysis and sugar metabolism in *SAG12-ipt* plants, which may support root growth under stress (Konishi *et al.*, 2005). Previous work has shown greater root growth and viability of *SAG12-ipt* plants under drought and heat stress conditions (Merewitz *et al.*, 2010, 2011; Y Xu. *et al.*, 2010). The present study also demonstrated a higher root to shoot ratio and lower root lipid peroxidation (MDA content). The abundance of IDH was greater in *SAG12-ipt* roots than in NT plants under non-stressed conditions and was decreased due to drought in NT roots, but not significantly changed in *SAG12-ipt* roots. IDH is an enzyme of the tricarboxylic acid (TCA) cycle, may be involved in N assimilation, has been found to be associated with leaf senescence, and its production of NADP could contribute to antioxidant defenses but is dependent on the specific isoform or cellular location (Corpas *et al.*, 1999). Previously, *SAG12-*

ipt plants were shown to maintain higher levels of IDH in roots under heat stress than NT plants (C Xu *et al.*, 2010). Inhibition of senescence may require more N for sustained chlorophyll and protein synthesis in maturing leaves. Since drought may restrict N uptake, and greater levels of N uptake have been associated with drought tolerance (Patrick and Wyatt, 1964; Foyer *et al.*, 1998), increased IDH prior to and during drought stress may allow *SAG12-ipt* to develop more efficient N and antioxidant metabolism for increased drought adaptability. However, the different isoforms of IDH could contribute to different metabolic functions or tolerance mechanisms; thus further work to identify changes in the different isoforms of this protein specifically in response to leaf senescence and drought in *SAG12-ipt* plants may be warranted.

Additionally, energy-producing enzymes such as ATP synthase subunits (root R39) in roots and 6PGDH subunits (leaf L31, root R21) were greatly increased in *SAG12-ipt* roots, but not in NT roots in response to water stress. 6PGDH functions in the oxidative phase of the pentose phosphate pathway, the alternative pathway to glycolysis, to generate NADPH, which serves as an energy source and plays a major role in preventing ROS by regulating glutathione peroxidase (Kruger and von Schaewen, 2003). The abundance of 6PGDH was also greater in *SAG12-ipt* under non-stressed conditions. Greater levels of glycolytic enzymes, sucrose synthase, and the potential for more energy production in the form of ATP and NADPH in roots of *SAG12-ipt* may contribute to improved energy production for sustained root growth and viability under the same degree of cellular water stress.

Changes in Proteins with Functions Related to Protein Synthesis

One of the mechanisms by which CKs prevents leaf senescence is through the promotion of protein synthesis (Chernyadev, 2005; Davies, 2010). A chloroplast elongation factor Tu (EF-Tu; leaf 120) and an RNA-binding protein (leaf L5) were reduced by drought stress in NT but not in *SAG12-ipt* leaves (Table 1, columns 2 and 3). A leaf mitochondrial EF-Tu (leaf L17) was increased in NT plants by drought stress but not changed in *SAG12-ipt* plants. The differential changes in chloroplast and mitochondrial EF-Tu, taken together with the differential regulation of photosynthetic and mitochondrial proteins (discussed in the energy category), may indicate that drought tolerance in *SAG12-ipt* leaves involves maintenance of photosynthetic protein synthesis with reduced levels of protein synthesis in mitochondria.

In roots, the abundance of a putative asparagine-tRNA ligase (root 57) accumulated more in *SAG12-ipt* relative to the change in NT in both the non-stressed and stressed conditions. Asparagine-tRNA ligase is an enzyme that catalyses the reaction determining the aminoacyl-tRNA activity state for alanine and aspartate metabolism and aminoacyl-tRNA biosynthesis. Conversion of the tRNA to the AMP form by the ligase can lead to asparagine synthesis. In regards to its possible association with the *SAG12-ipt* transgene, a delay in senescence has been linked to a delay in accumulation of asparagine and other free amino acids (Downs *et al.*, 1997). Aminoacyl-tRNA molecules are associated with other processes in addition to protein synthesis, such as the synthesis of porphyrin ring

structures, phospholipid synthesis, or peptidoglycan cross-linking (Mocibob *et al.*, 2010). Since this enzyme was more abundant under non-stressed conditions in *SAG12-ipt*, it could be involved in the CK biosynthesis promoted by the *SAG12-ipt* gene. Under water stress, this enzyme may be beneficial to *SAG12-ipt* roots by stimulating biosynthesis of these molecules. For instance, phospholipid and peptidoglycans could aid in membrane and cell wall stability. In addition, the direct product of the *SAG12-ipt* gene, iPa, is directly associated with tRNAs in translation, and derivatives of iPa may improve tRNA efficiency (Persson *et al.*, 1994). In general, elevated levels of proteins involved in translation could be beneficial for maintenance of protein synthesis under drought stress and be a factor in the reduced senescence in *SAG12-ipt* plants. Increased efficiency of protein synthesis under stressed conditions, when metabolic costs are high and restricted, may allow for increased metabolic functioning in *SAG12-ipt* roots.

Changes in Proteins involved in the Regulation of Protein Destination/Storage

In both leaves and roots, the abundance of PDI (leaf L3 and root 90) was maintained in *SAG12-ipt* plants during water stress, but was reduced by water stress in leaves and roots of NT plants. In roots, water stress caused an increase in the endoplasmin homologue precursor of HSP90 (root 86), Hsp70 cognate (root 88), and the proteasome subunit alpha type-7 (root R29), and a decrease in a mitochondrial processing peptidase (MPP; root 23). Increases in the abundance of these proteins were most pronounced in *SAG12-ipt* roots, whereas decreases were

more prominent in NT roots. Both PDI and a ferredoxin-nitrite reductase precursor were lower in *SAG12-ipt* than in NT plants under well-watered conditions. The abundance of root Hsp83 (R40) was increased by drought in response to water stress in *SAG12-ipt*, but a significant change did not occur in NT. Thus, improvements in drought tolerance of *SAG12-ipt* relative to NT could be related to increased protein chaperone and import function capabilities since Hsp90, Hsp70, and PDI are all involved in assisting protein folding (Georgopoulos and Welch, 1993). Similarly, MPP is involved in protein import to (Braun and Schmitz, 1997) and replacement of damaged proteins in the mitochondria during stress conditions (Taylor *et al.*, 2005).

Changes in Proteins Functioning within the Cellular Structure and Growth Category

One leaf protein in the cell structure category, a type IIIa membrane protein cp-wap13 (leaf L35), exhibited differential accumulation in response to water stress between the *SAG12-ipt* and NT plants. Cp-wap13 proteins are associated with the Golgi apparatus as well as cellulose biosynthesis in the cell wall, primarily in plasmodesmata, and are up-regulated by biotic stress (Shoresh and Harman, 2008). Their role in abiotic stress is unclear; however, in reference to its functions, increased levels of plasmodesmata proteins in *SAG12-ipt* plants could lead to enhanced root water transport properties. Root cell structure proteins such as actins (root R46, R47) were up-regulated in response to drought in both plant types, but R476 was increased more in *SAG12-ipt* roots. The abundance of

β-5 tubulin protein (Y186) increased in response to water stress only in *SAG12-ipt*

roots. Transcription of actin and tubulin structural proteins is highly hormonally

regulated, and their accumulation levels affect cell growth, size, and cellular

signalling under both non-stress and stressed conditions (Lang-Pauluzzi and

Gunning, 2000; Klyachko, 2003). Tubulin proteins are regulated by osmotic stress

(Komis *et al.*, 2002) and drought stress (Bagniewska-Zadworna, 2008), and are

differentially regulated in *ipt* bentgrass in response to heat stress (Y Xu *et al.*,

2010). Maintenance of cell structural proteins may also be related to root viability

(Klyachko, 2003; Bagniewska-Zadworna, 2008). Cell structural protein changes

in *SAG12-ipt* roots could be a response to the influence of the *ipt* gene on root

hormonal responses to drought, such as the ABA:CK ratio. Previously reported

root hormonal changes in *SAG12-ipt* plants, the promotion of root growth

(Merewitz *et al.*, 2010), and the changes in cell structural proteins suggest that

SAG12-ipt plants have a root hormonal status conducive to increase cell structural

integrity that stimulates root growth under drought stress conditions.

Protein Changes in the Signal Transduction Category

In roots, the abundance of three forms of GTP-binding proteins (root 29,

30, 31) was greater in NT plants under water stress relative to the well-watered

control conditions and compared with *SAG12-ipt* plants under water stress. GTP-

binding proteins are responsible for the regulation of G proteins, which control

many different cellular processes including cell division (Jones and Assmann,

2004). The difference in G protein accumulation could be related to the

differential CK and ABA content in roots of *SAG12-ipt* and NT plants, as reported

in Merewitz *et al.* (2010). The mechanism and function of several G proteins have

not been fully elucidated, but they may play a role in guard cell responses to ABA

and drought (Assmann, 2002; Perfus-Barbeouch, *et al.*, 2004). Thus, the role of

GTP-binding proteins in NT plant responses to water stress is unclear, but

increased activation or inactivation of G proteins by GTP-binding proteins could

be related to stress damage and ABA in NT plants. The abundance of 14-3-3E

was reduced by water stress in NT roots, whereas in *SAG12-ipt* roots it was

increased. The 14-3-3E proteins are involved in signal transduction processes

such as those that regulate cell elongation (Z Zhang *et al.*, 2010) and are

associated with enzymes involved in primary metabolism, many of which were

increased in *SAG12-ipt* roots relative to NT roots under stress, such as nitrate

reductase (NR), sucrose synthase, GS, and GADPH (Roberts *et al.*, 2002). Thus,

the maintenance of adequate levels of 14-3-3E protein may be a factor

contributing to maintenance of signalling capabilities under water stress in

SAG12-ipt plants.

Changes in Proteins Related to Stress Defense

The abundance of several antioxidant enzymes and chaperone proteins

was altered by water stress or the expression of the *SAG12-ipt* gene. The

abundance of 2-Cys peroxiredoxin (2-CP; leaf L11) decreased significantly in

response to water stress in NT plants, but did not change in *SAG12-ipt* plants. 2-

CP is an antioxidant enzyme that detoxifies hydroperoxides and peroxidized

lipids; it plays an important role in the protection of the photosynthetic machinery, particularly PSII (Baier and Dietz, 1999), and may be directly regulated by CKs (Rhee *et al.*, 2005). An increase in 2-CP content was found in *Arabidopsis* in response to elevated CKs (Lochmanova *et al.*, 2008), and tall fescue plants overexpressing 2-CP exhibited increased stress tolerance (Kim *et al.*, 2010). Mitochondrial root 2-CP is essential for root growth under stress in *Arabidopsis* (Dietz *et al.*, 2006). The maintenance of 2-CP and other antioxidants within *SAG12-ipt* plants could contribute to better physiological performance under water stress, as demonstrated by the lower EL and MDA content at the same level of international water deficit (47% RWC) relative to NT plants.

CAT is an antioxidant enzyme that converts harmful H_2O_2 into H_2O and O_2, and CAT levels decrease during leaf senescence (Dhindsa *et al.*, 1981). Under well-watered conditions, *SAG12-ipt* leaves accumulated a greater protein content of two isoforms of CAT (leaf 111, L23) and total activity (based on total protein content) relative to NT lines, suggesting that CAT may be involved in *SAG12-ipt* inhibition of natural leaf senescence. Under drought stress, CAT activity in *SAG12-ipt* plants was generally increased followed by a slight decline by 5% SWC, which was reflected in differential responses of accumulation of the CAT isoforms by two-dimensional PAGE. Greater CAT content of some isoforms and overall activity, together with the delay in decline of F_v/F_m, suggested that *ipt* plants exhibited a reduction in drought-induced leaf senescence. This is consistent with previous research indicating a negative correlation of leaf senescence and CAT activity under optimal growth conditions (Dhindsa *et al.*, 1981), and in *ipt*

tobacco, CAT was up-regulated by drought stress to a greater extent than in non-transgenic plants, and CAT remained more active for a greater duration of drought stress (Rivero *et al.*, 2007).

In leaves, SOD content differences between *SAG12-ipt* and NT leaves were not detected in either the well-watered or the drought-stressed condition; however, SOD activity was higher in *SAG12-ipt* leaves relative to NT leaves during both control and drought conditions. Water stress had a relatively minimal effect on the activity of SOD, which is consistent with previous reports that indicated great stability of SOD during senescence and drought (Dhindsa *et al.*, 1981). In roots, the abundance of SOD increased under water stress in NT plants (root 64); however, the activity of root SOD was greater in *SAG12-ipt* plants. Taken together, the results of both SOD and CAT activity and protein content suggest that greater activity of these enzymes in *SAG12-ipt* compared with NT may have compensated for costly antioxidant enzyme biosynthesis and thereby could contribute to greater root viability under stress. The general responses of increased antioxidant activity in bentgrass could be responsible for the lower lipid peroxidation in leaves and roots for *SAG12-ipt* plants under water stress.

Biochemical assays (Leshem *et al.*, 1979; Pauls and Thomson, 1982) and exogenous application of ZR to heat-stressed bentgrass (Liu and Huang, 2002) have implicated that CKs may play an indirect role in the maintenance of antioxidant systems. It is largely accepted that the relationships of CKs to antioxidant systems is due to the role of CKs in cellular signalling, which leads to

inhibition of senescence-promoting enzymes such as lipoxygenases (Brathe *et al.*, 2002) to slow the production of ROS caused by anabolic processes during senescence or stress. In addition, the results of MDA analysis are consistent with the potential that CKs may enhance the antioxidant system, resulting in a reduction of lipid damage by ROS, which has also been found in other *ipt* plant species (Qi-xian *et al.*, 2007). In *Pssu-ipt* tobacco, increased activity of PODs was also found under both non-stressed and drought stress conditions and was attributed to differences in peroxisome content between the wild-type and *ipt* plants (Synkova and Valcke, 2001). A strong antioxidant system under stress conditions plays a major role in stress tolerance of both leaves and roots of grass species (DaCosta and Huang, 2007; Wang and Jiang, 2007)

SUMMARY

This study compared proteins expressed differentially in *SAG12-ipt* transgenic bentgrass and NT plants subjected to the same level of internal leaf water deficit (47% RWC), which allowed for elucidation of metabolic processes controlling drought tolerance mechanisms that may be regulated by CKs. Major metabolic processes of drought tolerance regulated by CKs at the protein level included (i) energy production in both photosynthesis and respiration, primarily RuBisCO and GAPDH; (ii) synthesis of metabolites, primarily free amino acids such as methionine and glutamine; (iii) regulating protein synthesis, destination, and those with chaperone function, most notably enzymes in translation such as chloroplastic EF-Tu and PDIs; and (iv) maintenance of antioxidant responses, primarily with CAT and POD, and maintenance of proteins with roles in both energy production and signalling for stress defence such as GAPDH and IDH in leaves and roots of *SAG12-ipt* plants, which could be major factors contributing to the improvement in EL, F_v/F_m, and root viability. Reduced EL and MDA contents of leaves were associated with the greater activity and content of antioxidant enzymes, particularly those known to promote cell membrane stability such as 2-CP and CAT. In roots, the maintenance or accumulation of proteins involved in energy and N metabolism such as GS was associated with the increased root to shoot ratio and root viability observed in the *SAG12-ipt* plants.

It is worth noting that proteins which increased or maintained their content in *SAG12-ipt* lines may not always correlate with increased activity in their

respective biochemical pathway. For instance, the increase in photosynthetic enzyme subunits (leaf 28, 29, 30, and L7) under non-stress conditions does not seem to be correlated with greater levels of photosynthesis, since higher photosynthesis rates between *SAG12-ipt* and non-stressed NT plants have not been observed previously. However, generally, the greater content of photosynthetic enzyme subunit proteins was reflected physiologically by increased turf quality and F_v/F_m, lower EL and MDA content, root maintenance, and overall drought tolerance. Other potentially significant, but less well documented, protein changes occurred in response to CKs or drought in *SAG12-ipt* plants, such as membrane protein cp-wap13, 14-3-3E, DEAD-box helicase 2, and many proteins with unknown functions. Future evaluation of specific protein changes, particularly those less well documented in regards to CKs or drought stress or those with unknown functions, would be beneficial for more completely revealing the mechanisms of CK regulation of drought tolerance.

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TABLES AND FIGURES

Table 1 Relative leaf proteomic changes (%) in *SAG12-ipt* transgenic creeping

bentgrass relative to null transformant (NT) plant lines under well-watered

conditions (column 1) and drought stress conditions (column 4). Percent change

of proteins relative to the respective well watered control for NT (column 2) and

SAG12-ipt (column 3). Leaf proteins are listed by spot identification number

(SID). All listed values were statistically significant relative to the respective

comparison within a column. Asterisks indicate statistically greater differences

between numbers in columns 2 and 3. Proteins numbered as "L" followed by a

number were not previously identified in creeping bentgrass and therefore more

information is given about their characteristics. Statistical significance was based

on Fisher's LSD tests at ($P \leq 0.05$).

SID	Protein name	Transgene effect under non-stress conditions	Effects of drought (% change from control)		Differences between lines under drought
		(% change in watered *Sag12-ipt* from NT)	NT	*SAG12-ipt*	(% change in drought treated *Sag12-ipt* from NT)
Category 01 Metabolism					
3	Glycine decarboxylase P subunit/Victorin binding protein [*A. thaliana*]	ns	59.5	ns	ns
4	Alanine aminotransferase [*A. thaliana*]	ns	ns	ns	10.6

Table 1 - Effects of the transgene and drought stress on protein abundance in leaves of *SAG12-ipt* and NT creeping bentgrass

6	Aminomethyltransferase [*O.sativa* (japonica cultivar-group)]	ns	47.3	ns	ns
9	Methionine synthase [MS, *Hordeum vulgare* subsp. vulgare]	ns	-30.9	ns	ns
10	Methionine synthase [MS, *H. vulgare* subsp. vulgare]	ns	-27.1	89.9*	ns
11	Aspartate aminotransferase [*O. sativa*]	ns	ns	ns	61.9
12	Cell wall beta-glucosidase (β-D-glucan exohydrolase) [*Secale cereale*]	ns	ns	ns	-31.4
13	Cell wall beta-glucosidase (β-D-glucan exohydrolase) [*Triticum aestivum*]	ns	ns	ns	-75.0
14	Cell wall beta-glucosidase isoenzyme (Beta-D-glucan exohydrolase) [*T. aestivum*]	ns	-20.2	ns	-38.7
15	UDP-sulfoquinovose synthase [*O. sativa* ('japonica' group)]	ns	ns	-78.1	34.6
16	Adenosine diphosphate glucose pyrophosphatase [*H. vulgare* subsp. vulgare]	ns	-41.4	ns	71.7
L1	Possible: 3-hydroxy-3-methylglutaryl coenzyme A reductase [*Malus x domestica*] (40 kD, pI 8.0, gi|71159371)	ns	ns	ns	29.7
L24	Possible: Aspartate aminotransferase [*Pinus pinaster*] (53 kD, pI 7.5, gi|59932915)	ns	ns	-45.4	ns
Y110	Glycolate oxidase	ns	ns	-43.5	ns
Category 02 Energy					
18	RuBisCO large subunit [*Psathyrostachys fragilis* subsp. Fragilis]	ns	-20.2	45.2*	ns
20	RuBisCO large subunit [*Bulbine succulenta*]	ns	-30.9	ns	ns

26	RuBisCO large subunit [*Aira praecox*]	ns	ns	34.2	56.1
28	RuBisCO small subunit [*Avena maroccana*]	-24.6	ns	ns	ns
29	RuBisCO small subunit [*T. aestivum*]	35.0	ns	ns	ns
30	RuBisCO small subunit [*T. aestivum*]	50.6	ns	-16.1	ns
32	RuBisCO small subunit [*A. maroccana*]	ns	ns	ns	-41.3
34	RuBisCO small subunit [*Bromus catharticus*]	ns	ns	19.2	ns
36	RuBisCO small subunit [*T. aestivum*]	ns	ns	-30.5	ns
38	RuBisCO activase [*Nicotiana tabacum*]	ns	ns	-20.1	72.7
41	RuBisCO activase 1 [*Gossypium hirsutum*]	ns	-67.8	ns	ns
42	RuBisCO activase 1 [*G. hirsutum*]	ns	-32.0	ns	ns
44	Phosphoribulokinase (Phosphopentokinase) [*O. sativa* ('japonica' group)]	ns	-9.9	-33.1*	ns
11	GAPDH B, chloroplast precursor [*O. sativa* ('japonica group)]	117.0	ns	ns	ns
45	GAPDH, cytosolic [*O. sativa* ('japonica group)]	ns	-28.5	-21.2	ns
47	GAPDH B, chloroplast precursor [*O. sativa* ('japonica group)]	ns	49.8	ns	ns
49	GAPDH, cytosolic [*O. sativa* ('japonica group)]	31.8	-30.9	ns	26.0
51	GAPDH A, chloroplast [*O. sativa* ('japonica group)]	ns	ns	26.3	ns
54	GAPDH, cytosolic [*O. sativa* ('japonica group)]	ns	-37.4	48.3*	ns
55	GAPDH A, chloroplast [*O. sativa* ('japonica group)]	ns	-33.9	ns	ns
56	GAPDH A, chloroplast [*O. sativa*	ns	-69.1	ns	ns

	('japonica group)]				
57	GAPDH A, chloroplast [*O. sativa* ('japonica group)]	ns	ns	ns	37.0
58	GAPDH B, chloroplast precursor [*O. sativa* ('japonica group)]	ns	ns	ns	-40.7
60	Cytoplasmic fructose-biphosphate (FBP) aldolase [*O. sativa*]	ns	ns	-32.1	-14.2
61	Cytoplasmic aldolase [*O. sativa*]	ns	ns	ns	-27.8
63	Chloroplastic aldolase [*O. sativa*]	-23.5	ns	ns	ns
67	Cytoplasmic aldolase [*O. sativa*]	ns	ns	7.9	18.4
68	Ferredoxin-NADP(H) oxidoreductase [*T. aestivum*]	ns	-17.3	-22.6	ns
70	Triosephosphate isomerase, chloroplast precursor [*O. sativa* ('japonica group)]	ns	-69.9	ns	-63.3
72	Class III Alcohol dehydrogenase [*O. sativa*]	ns	ns	ns	22.3
73	Hydroxypyruvate reductase [*O. sativa* ('japonica group)]	ns	ns	22.7	ns
75	ATPase, β subunit [*H. vulgare*]	ns	-30.6	ns	ns
76	ATPase, β subunit [*H. vulgare*]	-35.7	ns	ns	ns
77	ATP synthase subunit β [*O. sativa* ('japonica group)]	-55.6	ns	ns	ns
78	ATP synthase ᵧ chain [*O. sativa* ('japonica' group)]	ns	-21.9	ns	38.6
79	Enolase (2-phosphoglycerate dehydratase) [*O. sativa* ('japonica' group)]	ns	-42.8*	-24.0	ns
80	Oxygen-evolving complex protein 1 (OEE1) [*A. thaliana*]	ns	-27.1	ns	ns
83	OEE2, chloroplast precursor [*Oryza sativa* ('japonica' group)]	ns	-17.3	13.8*	ns

88	PSAK (PS I Subunit K) [*A. thaliana*]	81.6	ns	ns	ns
89	PSI subunit N, chloroplast precursor (PSI-N) [*A. thaliana*]	ns	ns	ns	67.4
91	PS I subunit VII [*O. sativa* ('japonica' group)]	ns	ns	195.8	ns
92	Cytochrome b6-f complex iron-sulfur subunit, chloroplast precursor (Rieske iron-sulfur protein) [*A. thaliana*]	ns	-50.7	ns	ns
93	Aconitate hydratase, cytoplasmic (Aconitase) [*O. sativa* ('japonica' group)]	ns	162.8	ns	36.2
96	Carbonic anhydrase, chloroplast precursor	ns	103.0	ns	ns
L10	Light-harvesting complex I; LHC I [*H. vulgare*] (24kD, pI 8.1, gi|544700)	ns	ns	48.6	ns
L14	atp1 [*T. aestivum*] (55kD, pI 5.7, gi|81176509)	ns	-36.7	25.3*	47.3
L15	Isocitrate dehydrogenase [NADP], chloroplastic precursor (48kD, pI 6.2, gi|2497259)	ns	-42.2	-20.1*	ns
L18	PSI type III chlorophyll a/b-binding protein (29kD, pI 8.6, gi|430947)	ns	ns	80.1	ns
L19	Triosephosphate isomerase, cytosolic; (27kD, pI 5.4, gi|2507469)	ns	ns	-28.4	ns
L2	Possible: putative cytochrome c oxidase subunit II PS17 (2kD, pI9.6, gi|109892850)	ns	76.7	91.8*	18.7
L20	6-phosphogluconate dehydrogenase, decarboxylating [*Chlamydomonas reinhardtii*] (61 kD, pI8.4, gi|15225026)	ns	-43.1	ns	ns
L31	Putative phosphogluconate dehydrogenase [*O. sativa* 'japonica' group] (45kD, pI5.4, gi|55295906)	13.2	-20.2	-20.7	ns

L36	Glyceraldehyde 3-phosphate dehydrogenase β subunit [*A. thaliana*] (43kD, pI5.6,gi|336390)	71.9	ns	-39.3	ns
L4	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (33kD, pI 6.2, gi|120668)	ns	ns	67.6	ns
L6	Possible: ferredoxin [*Zea mays*] (41kD, pI 8.7, gi|162458489)	ns	-34.4	ns	ns
L7, 135	Photosystem I subunit VII [*Oryza sativa 'j*aponica' group] (8.9kD, pI6.5)	ns	ns	147.5	19.6
L9	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [*Avena sativa*] (18kD, pI 8.8, gi|4038695)	ns	ns	-33.7	ns
Y172	Chloroplast chlorophyll a/b-binding protein precursor [*Oryza sativa*]	ns	ns	125.1	ns
Category 03 Cell growth/division					
L16	Possible: DEAD-box ATP-dependent RNA helicase 2 -Arabidopsis thaliana (Mouse-ear cress) (46kD, pI 6.0, gi|109893655)	ns	26.9	ns	ns
Category 05 Protein synthesis					
120	Chloroplast translational elongation factor Tu [*O. sativa* 'japonica' group] 50kD, pI6.1)	ns	-50.9	ns	ns
L5	Putative RNA binding protein [*A. thaliana*] (43kD, pI 8.2, gi|3850621)	ns	-19.9	ns	ns
L17	Possible:mitochondrial elongation factor Tu [*A. thaliana*] (52 kD, pI 5.5,gi|1149571)	ns	57.6	ns	ns
L30	Possible: Elongation factor G, chloroplast precursor (ISS),[*Ostreococcus tauri*] (86kD, pI5.3, gi|116059008)	ns	ns	ns	-40.0

Category 06: Protein destination/storage					
101	Heat shock protein 70 [*Cucumis sativus*]	ns	ns	ns	-26.9
103	RuBisCO large subunit-binding protein subunit beta, (60 kDa chaperonin subunit beta)	ns	-33.9	ns	ns
L3	Putative protein disulphide isomerase (PDI) [*Brassica napus* var. napus] (26kD, pI 6.5, gi|45593261)	ns	-42.5	ns	ns
L34	OSJNBa0039C07.4 (HSP93 III) [*O. sativa* ('japonica' group)] 98kD, pI5.8, gi|38347158)	14.3	ns	ns	-39.5
Category 09 Cell structure					
L35	Type IIIa membrane protein cp-wap13 [*Vigna unguiculata*] (39kD, pI6.2, gi|2218152)	ns	ns	-33.5	ns
Category 11 Disease/defense					
109	Catalase-1 [*O. sativa* ('japonica' group)]	ns	ns	18.1	19.0
110	Catalase-1 [*O. sativa* ('japonica' group)]	ns	ns	-25.9	ns
111	Catalase-1[*O. sativa* ('japonica' group)]	58.7	45.7	ns	ns
114	Ascorbate peroxidase APX4 [*A. thaliana*]	ns	ns	ns	20.8
115	Ascorbate peroxidase APX7, chloroplastic [*O. sativa* ('japonica' group)]	ns	ns	ns	-23.6
116	Glutathione-S-transferase (GST) [*H. vulgare*]	ns	-40.6	ns	17.1
L11	2-Cys peroxiredoxin BAS1, chloroplast precursor [*T. aestivum*] (23kD, pI 5.7, gi|2829687)	ns	-31.3	ns	ns
L23	Possible: catalase (56kD, pI 6.7,	26.6	87.2	ns	ns

	gi|1705626)				
L37	Possible: ascorbate peroxidase [*A. thaliana*] (28kD, pI5.9, gi|555576)	ns	ns	-20.6	ns
Category 20 Secondary metabolism					
L22	Glycine decarboxylase P subunit [*Tritordeum* sp.] (111kD, pI 6.5, gi|2565305)	ns	-67.8	ns	ns
L27	S-adenosylmethionine synthetase (gi|3914019)	ns	-37.7	ns	ns
Unclear					
117	Unknown	ns	-27.1	ns	ns
118	Unknown	ns	-37.4	-15.5	ns
119	Unknown	ns	ns	ns	-59.9
122	Unknown	ns	35.3	30.9	-19.5
123	Unknown	ns	-37.7	-50.4	ns
127	Unknown	ns	ns	ns	15.0
131	Unknown	ns	ns	ns	69.3
137	Unknown	ns	ns	ns	69.3
139	Unknown	ns	-30.9	ns	ns
141	Unknown	ns	ns	22.2	ns
142	Unknown	ns	52.8*	27.7	19.6
148	Unknown	ns	ns	-26.2	44.1
L12	Unknown	ns	-68.1	261.2*	60.1
L26	Unknown	ns	ns	-33.1	ns
L32	Unknown	77.1	47.0*	-36.3	ns

Table 2 Relative root proteomic changes (%) in *SAG12-ipt* transgenic creeping bentgrass relative to null transformant (NT) plant lines under well-watered conditions (column 1) and drought stress conditions (column 4). Percent change of proteins relative to the respective well watered control for NT (column 2) and *SAG12-ipt* (column 3). Leaf proteins are listed by spot identification number (SID). All listed values were statistically significant relative to the respective comparison within a column. Asterisks indicate statistically greater differences between numbers in columns 2 and 3. Proteins numbered as "R" followed by a number were not previously identified in creeping bentgrass and therefore more information is given about their characteristics. Statistical significance was based on Fisher's LSD tests at ($P \leq 0.05$).

SID	Protein name	Transgene effect under non-stress conditions	Effects of drought (% change from control)	Effects of drought (% change from control)	Differences between lines under drought
		(% change in watered *Sag12-ipt* from NT)	NT	*SAG12-ipt*	(% change in drought treated *SAG12-ipt* from NT)
Category 01 Metabolism					
4	Cytosolic glutamine synthetase (EC 6.3.1.2) [*Populus alba x Populus tremula*]	ns	-4.5	92.0*	ns
6	Serine hydroxymethyltransferase	ns	-33.2	ns	ns

	(SHMT) (EC 2.1.2.1) [*Arabidopsis thaliana]*				
7	Nucleotide-sugar dehydratase [*Arabidopsis thaliana*]	9.7	ns	ns	-31.3
48	Phosphoserine aminotransferase (EC 2.6.1.52) [*O. sativa*]	ns	ns	12.0	ns
51	Plastidic ATP sulfurylase (APS) (EC 2.7.7.4) [O. sativa]	ns	191.0	ns	-27.1
71	Cytosolic glutamine synthetase [*Glycine max*]	ns	ns	ns	25.5
72	Cytosolic glutamine synthetase [*Populus alba x Populus tremula*]	ns	109.6	147.6*	73.4
73	Methionine synthase [*Hordeum vulgare* subsp. vulgare]	ns	-32.8	ns	ns
R21	Cytosolic 6-phosphogluconate dehydrogenase [*Oryza sativa*] (52kD, pI 6.5, gi|38426301)	ns	ns	-22.2	ns
R20	Possible: Os08g0459600 [*O. sativa* 'japonica'] (44D, pI 8.7, gi|115476758)	ns	-28.4	74.1*	ns
R26	Possible: UCW116, putative lipase [*H. vulgare* subsp. vulgare] (39kD, pI7.4, gi|118748148)	ns	ns	ns	27.4
R43	Possible: aspartyl-tRNA synthetase [*Zea mays*] (61kD, pI 5.9, gi|226505476)	ns	11.8	ns	ns
Category 02 Energy					
8	Cytoplasmic aconitate	ns	-28.5	-37.0	ns

	hydratase (EC 4.2.1.3) [*A. thaliana*]				
12	Pyruvate kinase (EC 2.7.1.40) [*Glycine max*]	ns	23.6	ns	ns
20	Ferredoxin-NADP reductase precursor (EC 1.18.1.2) [*Zea mays*]	ns	ns	-37.2	ns
21	Ferredoxin-NADP reductase precursor (EC 1.18.1.2) [*Zea mays*]	ns	81.7*	35.9	-35.1
52	Sucrose synthase (EC 2.4.1.13) Ss1 [*H. vulgare*]	ns	-43.8	ns	ns
53	Cytosolic GAPDH (phosphorylation) (EC 1.2.1.12) [*H. vulgare*]	14.2	35.5	ns	ns
54	GAPDH (phosphorylating) (EC 1.2.1.12) [*H. vulgare*]	ns	ns	72.4	ns
55	Cytoplasmic FBP aldolase (EC 4.1.2.13) [*O. sativa*]	ns	-29.7	-37.3	ns
78	Isocitrate dehydrogenase [NADP], chloroplast precursor	55.9	-17.7	ns	17.8
79	Isocitrate dehydrogenase [*O. sativa* ('japonica' group)]	31.3	ns	ns	ns
81	Triosephosphate isomerase, cytosolic [*T.aestivum*]	ns	ns	50.7	ns
82	Enolase (2-phosphoglycerate dehydratase) [*T. aestivum*]	ns	ns	38.7	ns
83	Enolase [*Oryza sativa ('*japonica' group*)*]	ns	ns	63.8	ns
R1	L-malate dehydrogenase (MDH) [*A. thaliana*] (42kD, pI 9.0, gi|15232820)	ns	ns	ns	-23.0

R3	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (33kD, pI 6.2, gi|120668)	ns	30.8	40.6	ns
R14	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (33kD, pI 6.2, gi|120668)	17.1	ns	ns	ns
R17	NADPH producing dehydrogenase of the oxidative pentose phosphate pathway [*Zea mays*] (53kD, pI 5.9, gi|162463282)	ns	-17.8	ns	-38.7
R21	Cytosolic 6-phosphogluconate dehydrogenase [*O. sativa*] (52kD, pI 6.5, gi|38426301)	ns	-64.4	ns	ns
R22	Ribulose-1,5-bisphosphate carboxylase, large subunit [*Didymosalpinx norae*] (52kD, pI 6.5, gi|1770216)	ns	54.0	79.7*	ns
R23	O-methytransferase 4 [*T. aestivum*] (38kD, pI 5.6, gi|145693798)	ns	10.8	ns	ns
R27	Ferredoxin-NADP reductase precursor [*Z. mays*] (36kD, pI8.4,) homologue	ns	106.3	94.8	ns
R39	ATP synthase beta subunit [*T. aestivum*] (59kD, pI 4.7, gi|525291)	ns	ns	148.0	77.3
R44	Ferredoxin-nitrite reductase precursor [*T. aestivum*] (66kD, pI6.9, gi|218963620)	-51.9	-57.8	ns	ns
Y165	Glucose-6-phosphate isomerase (GPI) cytoplasmic (62 kD, pI 6.96,	ns	ns	ns	34.7

	Accn P49105)				
Category 05 Protein synthesis					
57	Putative asparagine-tRNA ligase.(EC 6.1.1.22) [*O. sativa*]	12.9	-69.6	-50.5*	-18.0
Category 06 Protein destination/storage					
23	Mitochondrial processing peptidase α-chain (MPP) (EC 3.4.24.64)	ns	-52.7	-31.2	ns
86	Endoplasmin homolog precursor (HSP90) [*T. aestivum*]	ns	ns	52.3	51.0
88	70 kDa heat shock cognate [*Vigna radiata*]	ns	ns	121.1	ns
90	Protein disulfide isomerase (PDI) 3 precursor [*T. aestivum*]	-53.8	-16.4	ns	ns
R6	Os09g0505600 [*O. sativa* 'japonica'] (24kD, pI 6.4) (possible proteasome function)	ns	ns	-29.7	-48.9
R29	Possible: Proteasome subunit α type-7 (28kD, pI8.4)	ns	143.1	153.6	ns
R40	Possible: heat shock protein 83 [*A. thaliana*] (81kD, pI5.0)	ns	ns	35.5	39.6
Y153	Putative t-complex protein 1 theta chain [*O. sativa*]	ns	-69.0	-50.5	ns
Category 08 Intracellular traffic					
59	Ran (Small GTP-binding protein) (Ran2) [*O. sativa*]	ns	ns	ns	-18.5
Category 09 Cell structure					

26	Reversibly glycosylated polypeptide [*T. aestivum*]	ns	52.4	40.0	ns
R46	Actin [*Cleistogenes songorica*] (42kD, pI5.5)	ns	77.0	109.0*	134.0
R47	Actin-1 (42kD, pI 5.5)	ns	99.4	133.9*	ns
Y186	β-5 tubulin [*Triticum aestivum*]	ns	ns	103.7	35.9
Category 10 Signal transduction					
29	GTP-binding protein [*O. sativa*]	ns	49.4	ns	ns
30	GTP-binding protein beta chain homolog curled-leaved [*N. tabacum*]	ns	43.2	ns	-22.8
31	GTP-binding protein beta chain [*N. tabacum*]	ns	ns	ns	-7.8
R49	Possible: 14-3-3E [*H. vulgare* subsp. vulgare] (29kD, pI4.8)	ns	-17.5	31.2*	ns
Category 11 Disease/defense					
33	Probable peroxidase (EC 1.11.1) 1 precursor anionic [*Z. mays*]	ns	ns	ns	-32.2
34	Probable peroxidase (EC 1.11.1) 1 precursor anionic [*Z. mays*]	ns	27.9	ns	ns
64	Superoxide dismutase (EC 1.15.1.1) (Mn) 3.2 precursor [*Z. mays*]	ns	77.3	ns	ns
66	Sti (Stress inducible protein) [*Glycine max*]	ns	ns	-32.0	-20.8
R5	Ascorbate peroxidase [*H. vulgare* subsp. vulgare] (27kD, pI 5.8)	ns	79.2	46.2	-24.3

Category 20 Secondary metabolism					
38	dDTP-glucose 4-6-dehydratases-like protein [*A. thaliana*]	ns	-16.7	ns	ns
39	Adenosylhomocysteinase (EC 3.3.1.1) [*T. aestivum*]	ns	-35.3*	-54.4	ns
40	S-adenosylmethionine synthase (SAMS) (EC 2.5.1.6) [*A. thaliana*]	ns	-31.7	-27.2	ns
41	SAMS (EC 2.5.1.6) [*Dendrobium crumenatum*]	ns	-31.0	ns	ns
68	UDP-glucose 6-dehydrogenase (EC 1.1.1.22) [*Glycine max*]	38.3	-29.1	ns	ns
93	UDP-glucose dehydrogenase [*O. sativa* 'japonica' group]	ns	-13.9	ns	ns
R18	S-adenosylmethionine synthetase (43kD, pI 5.4)	ns	-30.0	ns	33.9
Category 12 Unclear					
46	Unknown	ns	51.7	ns	ns
47	Unknown	ns	-11.7	ns	ns
94	Os04g0650800 [*O. sativa* (*'*japonica*'* group*)*]	ns	-31.5	ns	ns
97	Unknown	ns	34.2	ns	-45.6
98	Unknown	ns	-3.1	-39.8*	ns
99	Unknown	ns	24.3	ns	ns
101	Unknown	52.9	ns	ns	ns
102	Unknown	ns	205.0	ns	ns
104	Unknown	ns	7.0*	-26.1	-48.6
R2	Putative r40c1 protein - rice [*O. sativa* 'japonica' group]	ns	117.9	ns	ns

	(42 kD, pI 6.2)				
R16	Unknown	-61.8	-56.3	ns	50.1
R19	unknown	ns	ns	25.5	ns
R28	unknown	ns	180.8	313.8*	ns
R30	unknown	ns	74.3	211.9*	ns
R31	unknown	ns	-32.4	-43.6	ns
R32	unknown	ns	115.8	ns	ns
R33	unknown	ns	ns	93.5	-41.1
R34	unknown	ns	ns	ns	-23.0
R48	unknown	ns	80.6*	69.0	14.5
R51	unknown	ns	ns	172.0	ns

Figure 1. Soil volumetric water content (SWC, %) measured using buried time domain reflectometry probes (20 cm) during the 14 d duration of water treatment of well-watered and drought stressed *SAG12-ipt* and NT plants. Vertical bars indicate LSD values where significant differences were detected ($P \leq 0.05$) for comparison between plant lines on a given treatment day.
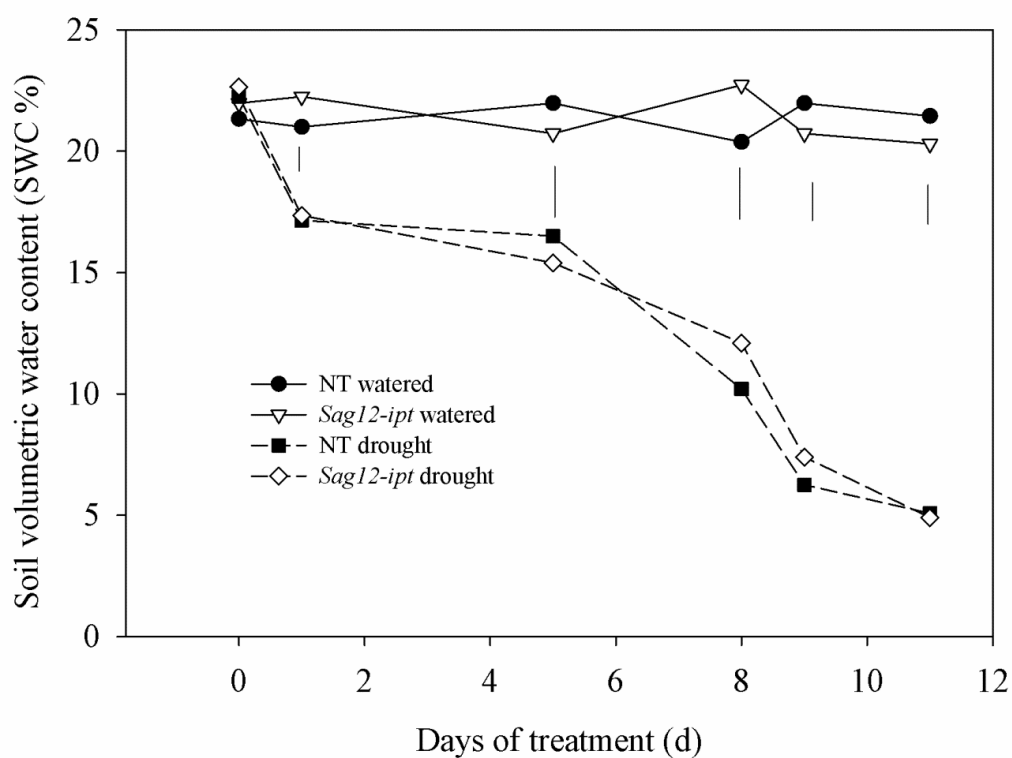
Figure 2. Leaf physiological responses of *SAG12-ipt* and NT leaves to drought stress conditions evaluated by measurement of A) turf quality, (TQ; 1-9 scale, with 1=completely desiccated and 9=healthy, turgid) B) photochemical efficiency (F_v/F_m) C) relative water content (RWC %) and D) electrolyte leakage (EL, %). Vertical bars indicate LSD values where significant differences were detected (*P* ≤ 0.05) for comparison between plant lines at a given soil water content (SWC %).
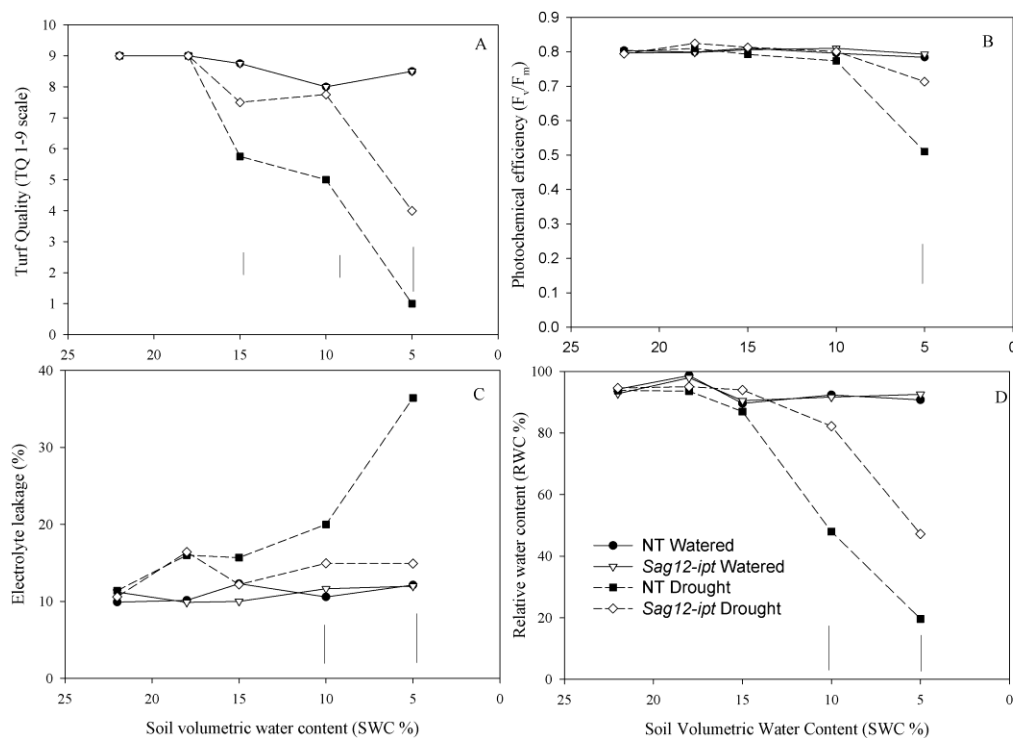
Figure 3. Leaf antioxidant activity responses of A) superoxide dismutase (SOD) B) peroxidase (POD) and C) catalase (CAT) in *ipt* transgenic creeping bentgrass (*SAG12-ipt*) compared to null transformant (NT) lines under drought stress. Vertical bars indicate LSD values where significant differences were detected (*P* ≤ 0.05) for comparison between plant lines at a given soil water content (SWC %).
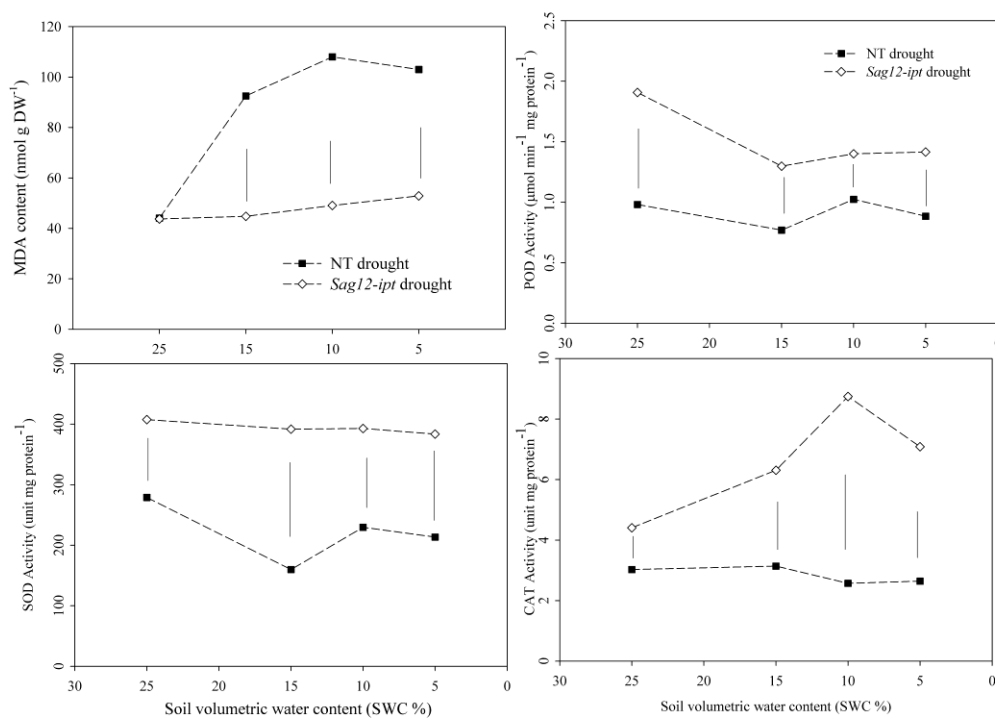
Figure 4. Root characteristics and enzyme activity assays of *ipt* transgenic creeping bentgrass (*SAG12-ipt*) compared to null transformant (NT) lines under drought stress as measured by A) root:shoot ratio B) root viability C) superoxide dismutase (SOD) and D) peroxidase (POD). Vertical bars indicate LSD values where significant differences were detected ($P \leq 0.05$) for comparison between plant lines at a given soil water content (SWC %).
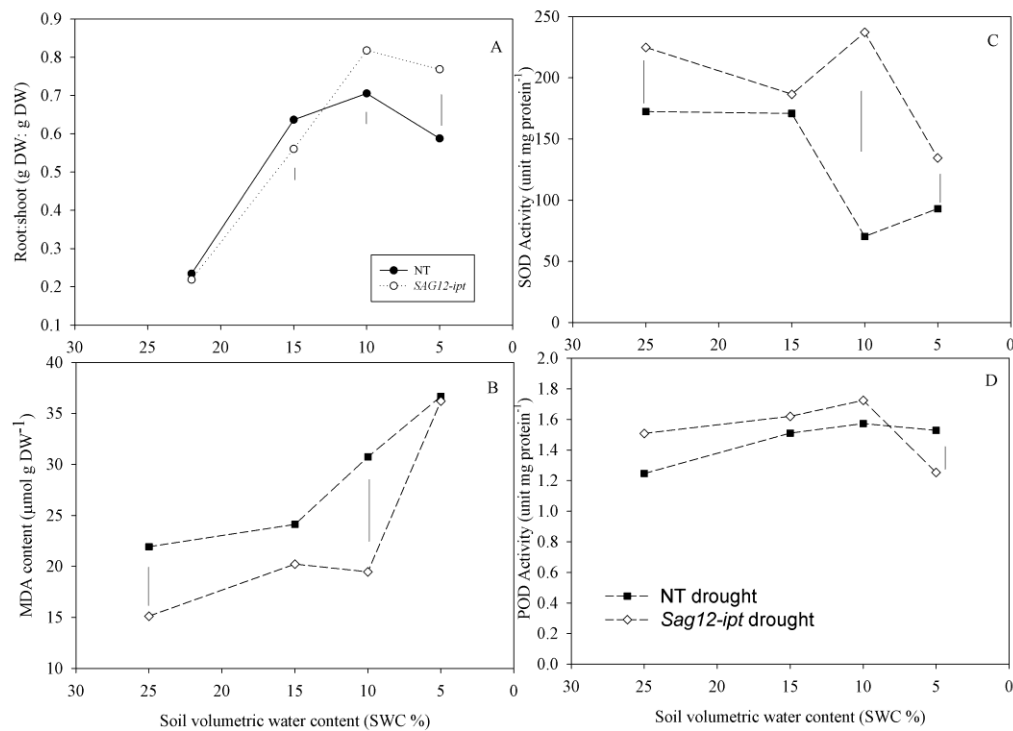
Figure 5. Representative gel image following two-dimensional polyacrylamide gel electrophoresis analysis of leaf protein extracts of null transformant (NT) and *ipt* transgenic creeping bentgrass (*SAG12-ipt*) exposed to water stress. Protein spots circled had differential accumulation due to water stress relative to the respective non-stressed control plant line (blue, greater accumulation; red, lower accumulation) ($P \leq 0.05$).
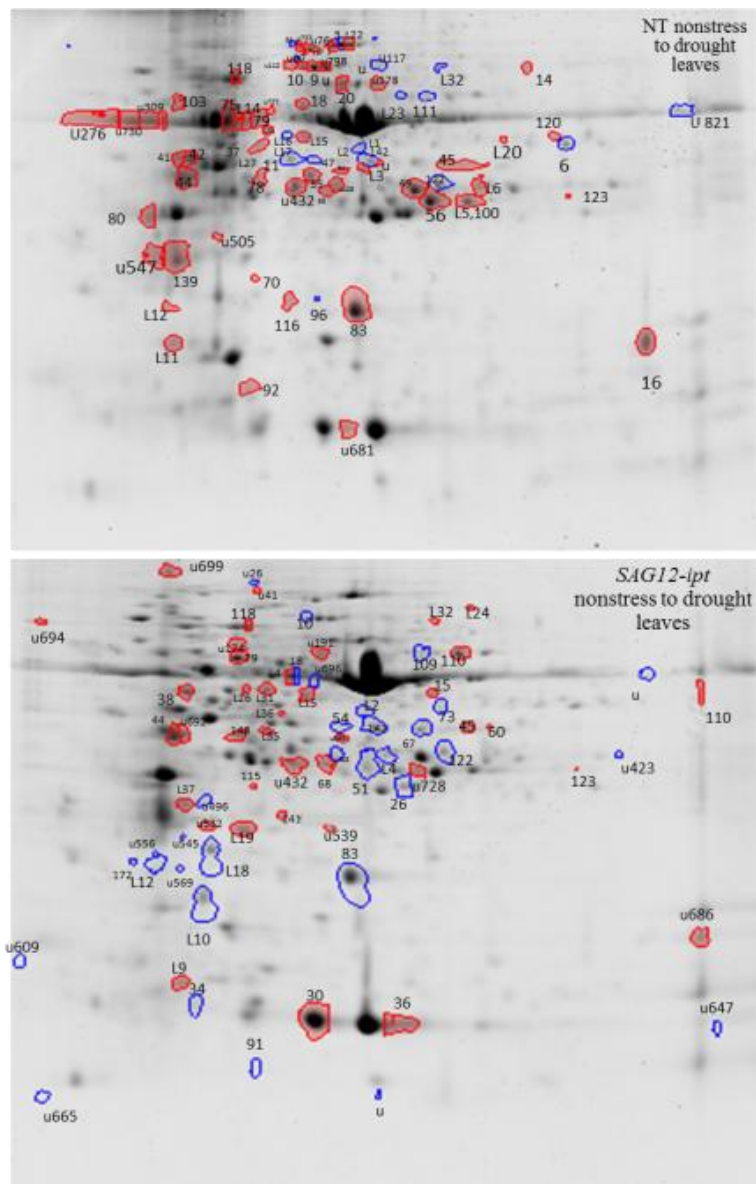
Figure 6. Four-way venn diagram comparing the number of proteins that exhibited a significant ($P \leq 0.05$) increase or decrease due to water stress in *ipt* transgenic creeping bentgrass (*SAG12-ipt*) compared to null transformant (NT) plant lines relative to the protein content of the respective well-watered control plants for leaves and roots. Overlapping regions of the circles indicate proteins that were regulated in either the same or opposite manner in the respective treatment whereas non-overlapping circles indicate proteins regulated in only that treatment.
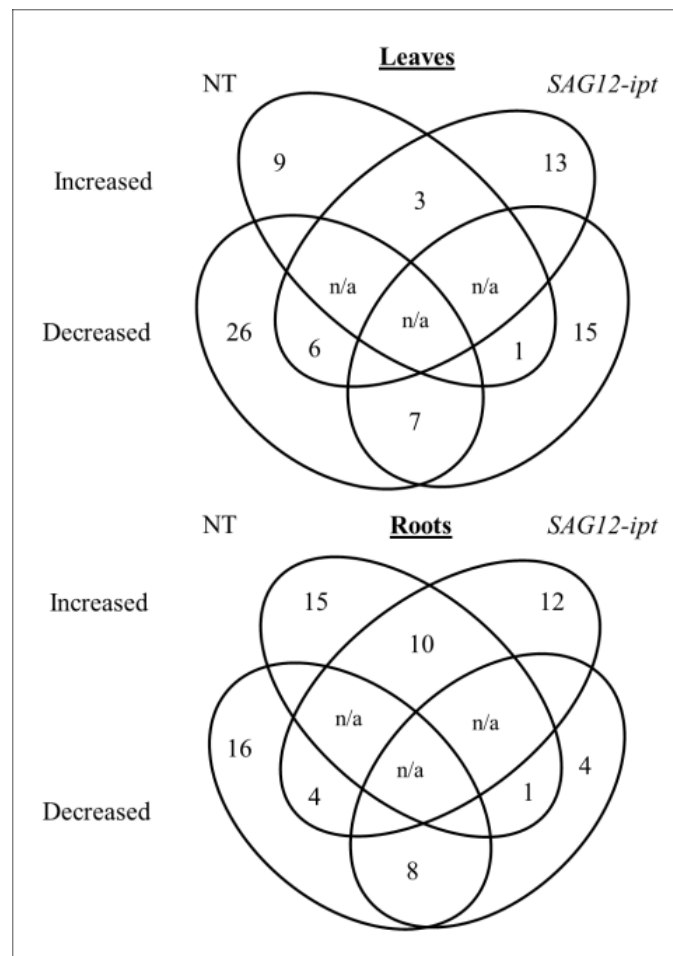
Figure 7. Percentages of proteins exhibiting significant differential expression (*P* ≤ 0.05) due to transgene expression or drought stress of *ipt* transgenic creeping bentgrass (*SAG12-ipt*) compared to null transformant (NT) lines within each functional category for A) leaves and B) roots.
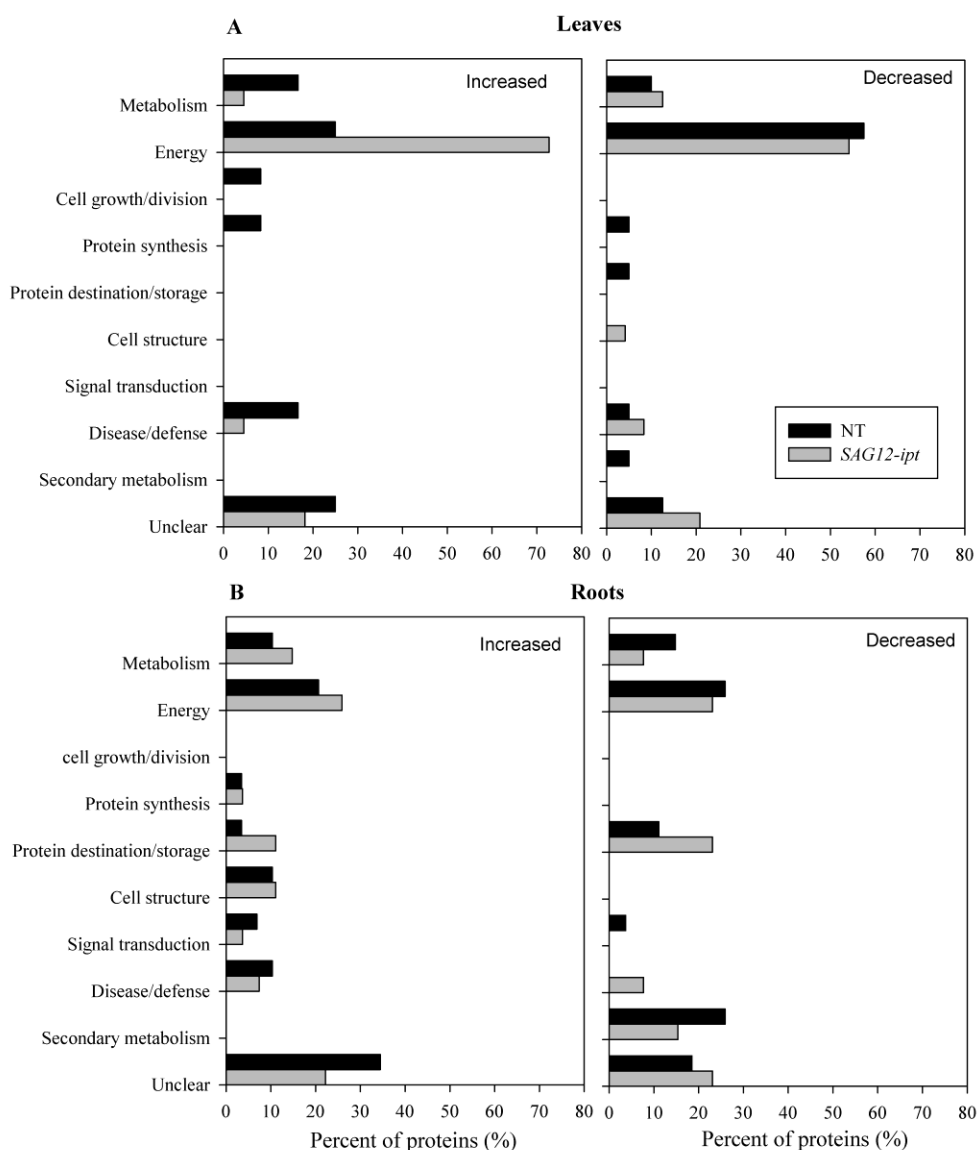
CHAPTER 4

ELEVATED CYTOKININ CONTENT IN IPT TRANSGENIC

CREEPING BENTGRASS PROMOTES DROUGHT TOLERANCE

THROUGH REGULATING METABOLITE ACCUMULATION

INTRODUCTION

Drought stress causes a cascade of physiological responses within plant cells, including changes in hormone metabolism (Davies *et al.*, 2010). The synthesis of cytokinin (CK), a growth-promoting hormone regulating cell division and various other growth and development events, typically declines in response to drought stress (Yang *et al.*, 2002; Kudoyarova *et al.*, 2006). A decline in CK production has been associated with leaf senescence and the growth inhibition of shoots induced by abiotic stresses, including drought stress. Molecular techniques such as transformation with a gene encoding *isopentenyl transferase* (*ipt*) to increase endogenous CK content under stress conditions have been found to be effective means to improve stress tolerance in various plant species, including monocot species such as rice (*Oryza sativa*) (Peleg *et al.*, 2011), tall fescue (*Festuca arundinacea*) (Hu *et al.*, 2005), and creeping bentgrass (*Agrostis stolonifera*) (Xu *et al.*, 2010; Merewitz *et al.*, 2010, 2011*a*, *b*). In *SAG12-ipt* creeping bentgrass (transformed with *ipt* controlled by a stress- or senescence-activated promoter), *ipt* expression resulted in increases in CK content, suppression of leaf senescence, and promotion of photosynthetic attributes, antioxidant scavenging, osmotic adjustment, and changes in root growth, which contributed to improved drought tolerance (Merewitz *et al.*, 2010, 2011*a*). These physiological changes characteristic of enhanced drought tolerance in the *ipt* plants were related to changes in the abundance and activities of various proteins in *ipt* plants relative to the null transformant (NT) plants during drought stress, particularly those proteins involved in photosynthesis, energy metabolism, and

antioxidant metabolism (Merewitz *et al.*, 2011*b*). Rivero *et al.* (2007, 2009) reported that improved drought tolerance in *ipt* transgenic tobacco (*Nicotiana tabacum*) was associated with increased photorespiration and antioxidant processes. Elevated CK content in *ipt* transgenic plants may induce various metabolic changes which could contribute to the improved drought tolerance. However, specific metabolites regulated by CK and involved in drought tolerance have not yet been fully evaluated.

Metabolomic analysis of various plant species has been previously used as a way to elucidate plant responses to various abiotic stresses (Dixon *et al.*, 2006). Knowledge of changes in the accumulation of specific metabolites aids in the understanding of metabolic pathways regulating stress tolerance responses, and could be used in future downstream applications such as gene identification and metabolomics-assisted plant breeding (Fernie and Schauer, 2008; Shulaev *et al.*, 2008). A combination of metabolic profiling, proteomic profiling, and physiological analysis is a powerful approach to increase the understanding of metabolic pathways controlling drought tolerance in various species (Bundy *et al.*, 2009). Previous studies have identified physiological processes and proteins regulated by CK under drought stress in *SAG12-ipt* creeping bentgrass (Merewitz *et al.*, 2010, 2011*a*, *b*). It is feasible to anticipate changes in the accumulation of metabolites resulting from *ipt* expression, which may contribute to CK-regulated drought tolerance. Therefore, the objectives of the study were to evaluate metabolite changes differentially exhibited between *ipt* and NT transgenic creeping bentgrass under well-watered and drought stress conditions in order to

identify potential drought tolerance mechanisms related to maintenance of CK

content under drought stress in a perennial grass species. Ultimately, the

metabolite changes revealed in this study can be used in conjunction with

previous studies on the effects of drought stress on physiological processes and

proteomic profiles for the identification of metabolic pathways of CK-regulated

drought tolerance.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Transgenic creeping bentgrass plants were produced using the *Agrobacterium* transformation method as described in Merewitz *et al.* (2010). Plant materials used for metabolite analysis by gas chromatography–mass spectrometry (GC-MS) were generated from the same experiment as for the proteomic analysis described in Merewitz *et al.* (2011*b*). Briefly, a null transformed line of 'Penncross' (NT) and the *SAG12-ipt* transgenic line (S41) were exposed to drought stress in an environmentally controlled growth chamber (Conviron, Winnipeg, Canada). Growth chamber conditions were set to maintain 20/15 °C (day/night) temperatures, 12 h photoperiod, 60% relative humidity, and 500 µmol m^{-2} s^{-1} photosynthetic photon flux density at canopy height. Plants were grown in PVC tubes (40 cm in height×10.16 cm in diameter) in a 1:1 fine sand:soil mix (fine-loamy, mixed mesic Typic Hapludult type soil).

Treatments and Physiological Measurements

Water treatments consisted of well-watered (control) or water completely withheld (drought) imposed on both NT and S41 plant lines (40 plants of each). Soil volumetric water content (SWC) was determined with the time domain reflectometry (TDR) method (Topp *et al.*, 1980) using a Trase TDR instrument (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Field capacity using this TDR system and a 1:1 soil was determined to be ∼25%. Measurements were

taken from 12 probes, four within the watered treatment and eight within the drought stress treatment (four replicates in each line), that were 20 cm long, three-pronged waveguide probes buried in the pot.

Leaf samples for metabolite analysis were taken from eight plants, four of each line, sampled at an SWC of 25, 10, and 5% in pots of both plant lines, which occurred over a period of 14 d. The treatment with 25, 10, and 5% SWC was designated as control, moderate drought stress (MD), and severe drought stress (SD), respectively. Leaf samples with a relative water content (RWC) of 47% were taken at 10% SWC for NT plants and 5% SWC for *SAG12-ipt* plants. Leaf RWC was evaluated to determine leaf hydration status for comparison of leaf metabolite changes at a given level of leaf RWC by the method of Barrs and Weatherley (1962). Leaf RWC was calculated based on fresh (FW), turgid (TW), and dry weights (DW) of ~0.1 g leaf samples. Leaf FW was determined on a mass balance immediately after being excised from the plants. Turgid weights were determined after soaking the leaves in de-ionized water for 12 h in a closed Petri dish and weighed immediately after they were blotted dry. Leaves were then dried in an 80 °C oven for at least 72 h prior to being weighed for DW. RWC was calculated using the formula: (FW–DW)/(TW–DW)×100. Other physiological parameters were measured to evaluate drought tolerance characteristics and were presented previously in Merewitz *et al.* (2010, 2011*a*, *b*).

Extraction of Polar Metabolites

Plant leaf tissue samples from NT and *SAG12-ipt* plants with a leaf RWC of ~47% were used for metabolic profiling. For extraction of metabolites, plant tissue was ground with a mortar and pestle in liquid N and frozen immediately at −80 °C until further analysis. The extraction protocol was modified from Roessner *et al.* (2000) and Rizhsky *et al.* (2004). For each sample, frozen leaves were ground to a fine powder with liquid nitrogen, and 25 mg of leaf tissue powders were transferred into 10 ml microcentrifuge tubes and extracted in 1.4 ml of 80% (v/v) aqueous methanol for 2 h at 200 rpm at ambient temperature. Ribitol solution (8 μl, 2 mg ml^{-1}) was added to each sample as an internal standard prior to incubation. Then, the tubes were placed in a water bath at 70 °C for 15 min, centrifuged for 30 min at 12 000 rpm, the supernatant was decanted to new culture tubes, and 1.4 ml of water and 0.75 ml of chloroform were added. The mixture was vortexed thoroughly and centrifuged for 5 min at 5000 rpm. A 2 ml aliquot of the polar phase (methanol/water) was decanted into 1.5 ml HPLC vials and dried in a Centrivap benchtop centrifugal concentrator. The dried polar phase was methoximated with 80 μl of 20 mg ml^{-1} methoxyamine hydrochloride at 30 °C for 90 min and was trimethylsilylated with 80 μl of MSTFA (with 1% TMCS) for 60 min at 70 °C. Ribitol (99%), methoxyamine hydrochloride, and *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were obtained from Sigma (St Louis, MO, USA). All other chemicals were analytical grade from the China National Pharmaceutical Group Corporation (Shanghai, China).

Gas Chromatography–Mass Spectrometry

The procedure for GC-MS analysis was modified from Qiu *et al.* (2007). The derivatized extracts were analysed with a PerkinElmer gas chromatograph coupled with a TurboMass-Autosystem XL mass spectrometer (PerkinElmer Inc., USA). A 1 μl aliquot of the extracts was injected into a DB-5MS capillary column (30 m×0.25 mm×0.25 μm, Agilent J&W Scientific, Folsom, CA, USA). The inlet temperature was set at 260 °C. After a 6.5 min solvent delay, the initial GC oven temperature was set at 60 °C; 1 min after injection, the GC oven temperature was raised to 280 °C at 5 °C min^{-1}, and finally held at 280 °C for 15 min. The injection temperature was set to 280 °C and the ion source temperature was adjusted to 200 °C. Helium was used as the carrier gas with a constant flow rate set at 1 ml min^{-1}. The measurements were made with electron impact ionization (70 eV) in the full scan mode (m/z 30–550). The metabolites detected were identified by Turbomass 4.1.1 software (PerkinElmer Inc., USA) coupled with commercially available compound libraries: NIST 2005, Wiley 7.0. For GC-MS results, compounds were identified based on retention time and comparison with reference spectra in mass spectral libraries. Response ratios were peak areas compared with the internal standard ribitol, and the peak areas were integrated with the Genesis algorithm. Relative content utilized throughout the manuscript refers to the ratio of peak area of the compouds to the total area of all other peaks within a given treatment.

Experimental Design and Statistical Analysis

Data were treated as appropriate for a split-plot design, with irrigation treatment as the main plots and plant materials as the subplots, with four replicates for each irrigation treatment and plant material. Effects of watering treatment, plant materials, and corresponding interactions were determined by analysis of variance according to the general linear model procedure of SAS using Fisher's protected LSD test at the 0.05 probability level (version 9.0; SAS Institute, Cary, NC, USA).

RESULTS

The effect of decreasing SWC on leaf RWC and drought damage to NT and *SAG12-ipt* creeping bentgrass plants has been reported previously (Merewitz *et al.*, 2011*b*). Briefly, decreasing SWC caused a decrease in leaf RWC to SD levels (47%) earlier in NT plants than in *SAG12-ipt* plants (10% SWC for NT and 5% SWC for *SAG12-ipt*). It was found that the maintenance of cellular membrane stability, increases in antioxidant capacity, and other physiological changes could be related to the better maintenance of RWC under drought stress by *SAG12-ipt* plants. Here, the results of metabolic analysis in both plant types are reported by comparing the content of a metabolite or group of metabolites over the course of drought stress (Figs 1– 6) and at an equivalent level of leaf water deficit (47% RWC; Fig. 7).

Metabolite Changes during Drought Stress: Amino Acids and Amine Derivatives

The total content of amino acids and amine derivatives declined in NT plants by 78% at MD and by 65% at SD, whereas the amino acid content in *SAG12-ipt* plants significantly increased by 19% under both MD and SD treatments (Fig. 1). The content of individual compounds within this group under well-watered conditions (25% SWC) and during drought stress (at 10% and 5% SWC) had differential patterns of accumulation (Fig. 2). Isoleucine and tryptophan did not have significantly detectable accumulation in response to either drought treatment or the transgene (data not shown). Under watered

conditions (25% SWC), the relative contents of the majority of amino acids were not significantly different between plant lines, with the exception of alanine and serine, which were significantly greater in NT plants compared with *SAG12-ipt* plants.

With increasing drought severity, the content of asparagine, glycine, and proline, increased in either or both transgenic and NT plants (Fig. 2A). Asparagine and proline increased in response to drought stress similarly in both plant lines (Fig. 2A); however, *SAG12-ipt* had a significantly greater content of proline than NT plants at MD and SD levels (Fig. 2) and at 47% RWC (Fig. 7). Glycine increased in response to drought under MD and declined back to the control level after SD in *SAG12-ipt* plants, but glycine content was not significantly different in NT plants from the control level (Fig. 2). Aspartic acid, dopamine, ethanolamine, serine, and threonine generally decreased in NT plants, whereas in *SAG12-ipt* plants a significant decrease was only detected for aspartic acid, threonine, and serine under drought stress (Fig. 2B). Under MD, ethanolamine content decreased by ~20% in *SAG12-ipt* leaves whereas it declined by ~80% in NT leaves. Alanine, γ-aminobutyric acid (GABA), pyroglutamic acid, and valine had an opposite pattern of accumulation in the plant lines (Fig. 2C). Alanine and GABA content both declined in NT plants but were increased in *SAG12-ipt* plants. For example, the initial contents of alanine were significantly different under control conditions (Fig. 2); however, drought stress

caused a decrease in alanine (67%) and GABA (80%) in NT plants, whereas there were increases in GABA (38.5%) and alanine (58%) in *SAG12-ipt* leaves (Fig. 7).

Carbohydrates and Other Modified Sugars

A total of eight major carbohydrates were identified in both plant lines at both levels of drought stress. Under watered conditions, a significantly greater amount of sucrose and a lower amount of floridoside was detected in *SAG12-ipt* leaves compared with NT leaves (Table 1). The carbohydrate content generally increased in response to drought stress in both plant lines (Fig. 1). In response to drought stress, the carbohydrates exhibiting a significant increase in both plant lines included glucose, fructose, and sucrose (Fig. 3A). Under SD, fructose and glucose content were ~50% greater in *SAG12-ipt* leaves than in the control condition, whereas there was no significant increase in NT leaves for fructose and not such a significant increase in glucose content for NT plants compared with the increase in *SAG12-ipt* plants. The difference in relative content of these metabolites relative to their respective controls was greater in *SAG12-ipt* plants than in NT plants at the same level of leaf water deficit (Fig. 7).

Galactose and maltose were less significantly impacted by drought stress or *ipt* expression; however, their content increased due to SD stress in *SAG12-ipt* leaves, whereas no significant increase was detected in NT leaves at any level of drought. Carbohydrates exhibiting a decrease in content due to drought stress in NT plants included floridoside (93%), ribose (57%), and melibiose (78%),

whereas there was no significant decline in floridoside, only 31% for ribose, and only 56% for melibiose in *SAG12-ipt* plants. Melibiose exhibited an increase in response to MD followed by a decline under SD in *SAG12-ipt* plants, whereas only a decrease in melibiose content occurred in response to drought stress in NT plants (Fig. 3B). At the same level of leaf water deficit, fructose, galactose, glucose, and sucrose were increased or maintained better in *SAG12-ipt* leaves since the difference in relative content relative to their respective controls was greater for *SAG12-ipt* plants compared with NT plants (Fig. 7).

Organic Acids and Alcohols

A total of 19 organic acids and four organic alcohols were detected in the leaves of both plant lines. Aconitic acid, malonic acid, glyceric acid, and shikimic acid were detected, but not significantly affected by either *ipt* expression or drought stress. Fumaric acid, glycolic acid, methylmaleic acid, threonic acid, PAME (phosphoric acid monomethyl ester), and succinic acid increased in response to drought stress but were not significantly different between plant types (data not shown). Total organic acid content exhibited a significant increase in response to drought stress in both plant lines, but under MD for *SAG12-ipt* plants and under SD for NT plants (Fig. 1). Of the organic acids, 2-keto-gluconic acid (2-KGA) and itaconic acid content increased under drought stress in NT, but not in *SAG12-ipt* plants. Malic acid content gradually increased with the increase in drought intensity, but the extent of increase was the same at both levels of water deficit for *SAG12-ipt* plants and lower than in NT plants under SD. Oxalic acid

content increased under MD for NT plants and under SD for the *SAG12-ipt* plants compared with their respective well-watered controls (Fig. 4). Hexanoic acid and pyruvic acid content decreased with drought stress for both plant lines, but occurred later in *SAG12-ipt* than in NT plants (Fig. 5A). Organic acids oppositly accumulated between the plant lines, including citric acid and lactic acid. The content of citric acid decreased with drought stress in NT but increased in *SAG12-ipt* plants under SD, and lactic acid declined significantly in NT plants but not significantly in *SAG12-ipt* plants.

Total organic alcohol content was significantly lower for NT plants under both MD and SD compared with the control, but no significant changes occurred in *SAG12-ipt* plants (Fig. 1). Changes in organic alcohols in response to drought stress in both plant lines included a decrease in the content of glycerol for NT plants and myo-inositol for both plant lines. Myo-inositol decreased significantly earlier in NT plants than in *SAG12-ipt* plants; the level of myo-inositol was ~50% lower in NT plants under MD but was not significantly different from the control condition in *SAG12-ipt* plants under MD. In response to SD, glycol and glycerol-3-phosphate were significantly increased in *SAG12-ipt* leaves, but not in NT leaves (Fig. 6).

DISCUSSION

Increased endogenous CK content through *ipt* transformation has been found to be associated with improved drought tolerance in various plant species (Clark *et al.* 2004; Zhang *et al.*, 2010; Peleg and Blumwald, 2011), including creeping bentgrass (Merewitz *et al.*, 2010, 2011*a*, *b*). The improvements in drought tolerance were observed through evaluation of several physiological parameters at an equivalent level of leaf water deficit. For example, *SAG12-ipt* plants were better able to maintain chlorophyll content (Merewitz *et al.*, 2010, 2011*a*) and photochemical efficiency (Merewitz *et al.*, 2010, 2011*a*) than NT plants under drought stress. Additionally, an elevated CK content was previously found to effect the concentration of other hormones such as abscisic acid (ABA) (Merewitz *et al.*, 2010) and protein content such as antioxidant enzymes (Merewitz *et al.*, 2011*b*). Thus, the changes in metabolites discussed here could be due to CK content directly or a secondary effect caused by the changes in other signalling agents, hormones, or other biochemical pathways. Despite the well-recognized physiological changes induced by CK, changes in CK-regulated metabolites in relation to drought tolerance are not well documented. Results from the present study suggest that *SAG12-ipt* creeping bentgrass were able to maintain higher levels of various metabolites or exhibited less decline in the accumulation of some metabolites under drought stress, compared with NT plants, and the differential regulation of major metabolites may reflect the influence of CK on the metabolic pathways associated with improved drought tolerance, as discussed below.

Total carbohydrate content increased under drought stress in both *SAG12-ipt* and NT creeping bentgrass leaves. In response to drought, inhibition of growth prior to cessation of photosynthesis and the hydrolysis of complex storage carbohydrates can contribute to an increase in the content of simple carbohydrates (Hsiao, 1973; Chaves, 1991; Spollen and Nelson, 1994). The accumulation of sugars in response to drought is frequently cited to be an important mechanism of plant adaptation to drought, particularly due to their role in maintaining cellular hydration by osmotic adjustment (Bray, 1997). Creeping bentgrass leaves that were able to maintain greater levels of carbohydrates under drought stress were found to be more tolerant to dehydration (DaCosta and Huang, 2006). In this study, *SAG12-ipt* creeping bentgrass was generally able to maintain a greater amount of total carbohydrates than NT plants. This finding could be related to the more active photosynthetic activities and increased level of proteins involved in light harvesting and carbon fixation, as well as other proteins involved in sugar metabolism under drought stress conditions due to elevated CK (Merewitz *et al.*, 2010, 2011*a*, *b*). CK deficiency in plants has been shown to cause a decrease in free sugars such as sucrose, glucose, and fructose, with a concomitant increase in starch content in shoot sink tissues of tobacco (*Nicotiana tobacum*; Werner *et al.*, 2008).

The accumulation of sucrose, galactose, glucose, fructose, and maltose was more apparent in *SAG12-ipt* than in NT plants in response to drought stress. Sucrose content was significantly greater under well-watered conditions and under drought stress in *SAG12-ipt* plants compared with NT plants. Peleg *et al.*

(2011) found the sucrose and starch content to be significantly higher in *ipt* rice (*Oryza sativa* L.) leaves under water stress, which was related to the finding that several genes for sugar transporters and starch synthesis were differentially regulated in *ipt* rice relative to the wild type. Rizhsky *et al.* (2004) found that glucose and fructose were accumulated under drought stress in *Arabidopsis*. Similar results were found in a metabolomics study of perennial ryegrass (*Lolium perenne* L.) under water stress, with a greater amount of glucose, fructose, maltose, and other carbohydrates in the more drought-tolerant cultivar (Foito *et al.*, 2009). In potato (*Solanum tuberosum*), galactose was a primary metabolite accumulated to a greater extent in a drought-tolerant cultivar relative to a sensitive one at similar levels of RWC (Evers *et al.*, 2010). It is known that carbohydrates of primary importance in drought response can be species specific (Gupta and Kaur, 2000). For instance, in *ipt* transgenic rice, mannitol and trehalose transporters were up-regulated by *ipt* (Peleg *et al.*, 2011), but these sugars were not detected in the perennial grass species in the present study. In grape (*Vitis vinifera* 'Cabernet Sauvignon'), glucose and malate were the primary carbohydrates up-regulated by drought stress (Cramer *et al.*, 2007). Ivic *et al.* (2001) did not see a significant alteration in sucrose content related to the *ipt* gene in sugarbeet (*Beta vulgaris* L.). Nevertheless, changes in CK content may alter accumulation of different forms of carbohydrates, leading to improved drought tolerance, although not all species may exhibit accumulation of the same types of carbohydrates in response to increased CK.

Other carbohydrates differentially regulated between NT and *SAG12-ipt*
plants were melibiose and floridoside. Melibiose is a disaccharide consisting of
galactose and glucose moieties and is known to be involved in the promotion of
cold tolerance (Sharma *et al.*, 2005). During drought stress, melibiose is often
below the detection limit in some species (Evers *et al.*, 2010; Legay *et al.*, 2011),
and therefore limited information is available regarding its involvement in
drought response or hormonal regulation. In *SAG12-ipt* leaves an increase in
melibiose content was detected under MD and then a significant decrease
occurred under SD, whereas melibiose content in NT plants decreased
consistently with increasing stress severity. A similar increase in melibiose
content followed by a decrease during drought stress was found only in stress-
tolerant bermudagrass (*Cynodon dactylon*) under heat stress, but not in stress-
sensitive Kentucky bluegrass (*Poa pratensis*; Du *et al.*, 2011). Under watered
conditions, a significantly lower amount of floridoside was detected in *SAG12-ipt*
leaves compared with NT leaves (Fig. 3B), and the same result was reported by
Du *et al.* (2011) in stress-tolerant bermudagrass. This could be related to a lower
amount of natural leaf senescence, since leaf senescence stimulates the
breakdown of more complex carbohydrates and starches to cause an increase in
low molecular weight sugars for energy availability (Crafts-Brandner *et al.*,
1984). Elevated endogenous CK content and the inhibition of senescence caused a
reduction in energy reserve remobilization in *ipt* tobacco (Cowan *et al.*, 2005). In
algae (*Rhodophyceae* sp.), floridoside is a photoassimilate serving as a ready
available energy reserve that can be a quick source of energy since it can be easily

cleaved to release glucose (Kremer, 1978). The roles of floridoside in plant stress responses or due to altered CK content, particularly in perennial grasses, are unknown.

The amino acids and amine derivatives asparagine and proline were increased by drought stress in both NT and *SAG12-ipt* leaves, whereas alanine, GABA, and valine increased only in *SAG12-ipt* plants. Other compounds, such as aspartic acid and serine, showed decreases due to drought stress in both plant types, or were maintained better by exhibiting less of a decline or no decline in *SAG12-ipt* leaves, such as ethanolamine and dopamine, respectively. Therefore, the total amino acid content exhibited an increase in *SAG12-ipt* plants but a decrease in NT plants. Free amino acids are known to accumulate in response to drought stress in various plant species, and drought-tolerant plants generally accumulate more free amino acids than drought-sensitive plants, although a few exceptions have been found (Rai, 2002). Proline accumulation due to drought stress has been studied extensively and is known to exhibit numerous beneficial functions in the stress defence response including osmotic adjustment, stabilization of cellular structures and membranes, and free radical scavenging (Cramer *et al.*, 2007; Szabados and Savoure, 2009). Dobra *et al.* (2010) found that tobacco plants expressing a transgene to increase endogenous proline content were able to mitigate drought-induced reductions of CK to a greater extent under drought stress than non-transgenic plants with lower levels of proline. In their study, proline content did not significantly alter bioactive CKs under non-stressed conditions. Thomas *et al.* (1995) found that the act of exogenously applying CK

to *ipt* transgenic tobacco plants may have elicited a slight osmotic stress response which could thereby increase proline content. Therefore, it was unclear whether an increase in proline content was directly related to elevated CK content or was indirectly caused through osmotic adjustment. It has been noted that the CK 6-benzylaminopurine delays leaf senescence, and a concomitant increase in proline content was observed (Alvarez *et al.*, 2008), but conflicting results have been found on the direct effect of benzylaminopurine and other CKs on proline biosynthesis genes (Hu *et al.*, 1992). The interaction between CK and proline content could also be related to the accumulation of carbohydrates such as glucose, fructose, mannitol, and, most notably, sucrose, since these carbohydrates have been found to play a role in increasing proline accumulation (Kishor *et al.*, 2005). In the present study, proline content increased to a greater extent in *SAG12-ipt* plants than in NT plants during drought stress, which could enhance osmotic adjustment and other stress defence mechanisms for plant tolerance to drought stress. However, whether proline accumulation in *SAG12-ipt* plants is due to direct effects of elevated CK content or is a result of an increase in carbohydrate content such as of sucrose or an osmotic response is unclear. Thus, the association of elevated CK content with proline accumulation in *SAG12-ipt* plants may be worth further investigation.

Several other free amino acids accumulated differentially in *SAG12-ipt* and NT leaves under drought stress. Asparagine exhibited an accumulation in response to drought in both *SAG12-ipt* and NT plants, but had a more marked increase in *SAG12-ipt* plants when both plants experienced the same level of leaf

water deficit (47% RWC). Asparagine content has been found either to increase or to decrease in response to drought, depending on the plant species and stress severity (Stewart and Larher, 1980; Aranjeulo *et al.*, 2011). Previously it was found by proteomic analysis of the same creeping bentgrass plants that several proteins involved in amino acid metabolism had differential expression patterns between NT and *SAG12-ipt* plants. For instance, the leaf proteins methionine synthase (MS), *S*-adenosylmethionine synthetase (SAMS), aspartate and alanine aminotransferases, and the root asparagine-tRNA ligase accumulated more or were degraded less in *SAG12-ipt* relative to the change in NT in during drought stress conditions (Merewitz *et al.*, 2011*b*). Changes in MS, SAMS, and aminotransferases could play a role in the altered accumulation pattern of various amino acids due to differential fluxes through the various amino acid synthesis and degrative pathways and play a role in osmotic adjustment (Bohnert and Jensen, 1996). Asparagine-tRNA ligase is an enzyme that catalyses the reaction determining the aminoacyl-tRNA activity state and has been linked to the regulation of expression of asparagine synthetase, and could lead to asparagine synthesis by feedback mechanisms, or function in other non-ribosomal pathways (Arfin *et al.*, 1977; Mocibob *et al.*, 2010). In addition, asparagine accumulation has been linked to a delay in leaf senescence (Downs *et al.*, 1997). Thus, the increased content of asparagine and aminoacyl-tRNA could be related to the increased endogenous CK and to the improved drought tolerance of *SAG12-ipt* plants. However, this mechanism is still unclear. Valine exhibited a large increase in *SAG12-ipt* leaves in response to drought, but did not change significantly in NT

leaves. Metabolic profiling of pea (*Pisum sativum* L.) conducted by Charlton *et al.* (2008) reported that valine along with leucine, isoleucine, and threonine were important metabolites exhibiting a large degree of accumulation in response to drought stress. Pyroglutamic acid (also known as 5-oxoproline) is an uncommon amino acid and is not well documented in drought stress response; however, it may be associated with freezing tolerance in *Arabidopsis* (Korn *et al.*, 2010). As an intermediate, pyroglutamic acid is a precusor to the synthesis of glutamate (Ohkama-Ohtsu *et al.*, 2008) and proline (Jones, 1985). Glycine is a proteogenic amino acid that functions in the biochemical pathway as a precursor leading to purines such as CKs and other porphyrin structures (Mok and Mok, 1994), and drought-responsive metabolites such as glycine betaine (Weretilnyk *et al.*, 1989) and glycine may act as a signalling molecule (Dubos *et al.*, 2003; Bouche and Fromm, 2004). Glycine is also a predominant precursor of the antioxidant compound glutathione and may result from an increase in photorespiration, which has been attributed to protection of the photosynthetic apparatus during drought stress (Rivero *et al.*, 2009, and references therein). The accumulation of the amino acids such as valine, pyroglutamic acid, and glycine could contribute to improved drought tolerance in *SAG12-ipt* plants as a source of precursors for key metabolic pathways regulating drought tolerance.

Alanine and GABA were differentially regulated between the plant lines since they increased in response to drought in *SAG12-ipt* leaves, but decreased in NT leaves. Increased alanine due to drought stress has been detected in other plant species. In rapeseed (*Brassica napus*), the accumulation of alanine was due to an

increase in the precursors pyruvate and glutamate, and not due to enzymes in the alanine biosynthetic pathway (Good and Zaplachinski, 1994). The same mechanism may be occurring in creeping bentgrass because a clear trend in accumulation of alanine aminotransferase was not observed previously by proteomic analysis (Merewitz *et al.*, 2011*b*). Several studies have shown that GABA accumulates in response to drought stress (Raggi, 1994; Bor *et al.*, 2008). GABA is a non-protein amino acid that may be a signalling molecule in plants involved in regulating numerous stress response mechanisms such as the carbon:nitrogen balance, osmotic potential, free radical scavenging, pH regulation, plant growth, and root development (Kinnersley and Turano, 2000; Bouche and Fromm, 2004). Specific to drought stress, GABA may function primarily as an osmolyte (Shelp *et al.*, 1999). However, studies evaluating GABA content have demonstrated a greater GABA content in drought-sensitive compared with drought-tolerant cultivars (Kinnersley and Turano, 2000), whereas other studies have found GABA receptor genes to be expressed more in drought-tolerant cultivars than in sensitive ones (Guo *et al.*, 2009). In regards to GABA as a signalling molecule, it has long been known that GABA interacts with various hormones in animals but has only recently been indicated to have this function in plants (Bouche and Fromme, 2004); therefore, the role of GABA in the drought response and its relationship to CK remain to be elucidated. This study may provide some evidence that CK signalling may alter the flux through the GABA shunt pathway or may be associated with changes in oxidative status or other signalling pathways. The differences in GABA content in *SAG12-ipt* plants

compared with NT plants could be related to differences in the content of GABA
precursors (pyroglutamic acid and proline), differences in CK, growth habits, or
osmotic adjustment, but the specific implications of GABA accumulation cannot
be directly concluded from this study alone. Future work focused on how CK
signalling pathways such as via calmodulin (Brault and Maldiney, 1999; Müller
and Sheen, 2007) may relate to the shift in GABA and alanine content may be
warranted. In general, the differential abilities of NT and *SAG12-ipt* plants to
maintain content at the control level, or actually to increase levels of various free
amino acids under drought stress, could be related to CK accumulation and may
be a factor promoting drought-enhanced tolerance in *SAG12-ipt* plants.

Of all the compounds in the amino acid and amino acid derivative
category, that exhibited a decrease in content in response to drought,
ethanolamine (ETA) and dopamine were less responsive to drought stress in
SAG12-ipt plants than in NT plants. ETA is primarily found in plant membranes
as the head group of polar lipids. Maintenance of ETA during drought stress could
be beneficial to *SAG12-ipt* plants since ETA-containing lipids inhibit the activity
of phospholipase D reactions. Phospholipases break down lipid membrane
bilayers and re-organize lipid saturation and structure (Austin-Brown and
Chapman, 2002). Additionally, ETA is a precursor to stress-protective compounds
such as glycine betaine and has been found to accumulate due to abiotic stress in
other plant species, protecting cellular membranes from stress damage (Suzuki *et
al.*, 2003). It has been found that *SAG12-ipt* plants with the ability to maintain
ETA content under drought stress exhibited less membrane damage, as

demonstrated by reduced electrolyte leakage, compared with NT plants (Merewitz *et al.*, 2010). Dopamine is a catecholamine regulating various growth and development processes and affecting carbohydrate metabolism and the actions of plant hormones such as ethylene (Dai *et al.*, 1993; Świędrych *et al.*, 2004; Kulma and Szopa, 2007). The synthesis of catecholamines is regulated by stress conditions, but the role and actions in stress responses are not well understood (Kulma and Szopa, 2007). Previously CK compounds have been shown to inhibit dopamine-13-hydroxylases (Abdelnour-Esquivel *et al.*, 1992). It is unknown whether the maintenance of dopamine content in *SAG12-ipt* plants may be due to less drought damage or a reduction in the activity of degrading enzymes in plants with elevated CK content.

The organic acids citric, oxalic, and malic acids may be important metabolites regulated by CK content since these compounds were maintained to a greater extent or were increased by drought stress in *SAG12-ipt* leaves (Fig. 7). Citric acid is a key intermediate of the citric acid (TCA) cycle where it is created from incoming pyruvate and acetyl Co-A molecules. The maintenance of citric acid content could be related to a differential flux through the TCA pathway in *SAG12-ipt* plants under drought stress. Oxalic acid is also known to be a source of defence against biotic stress (Punja, 2001). In grass pea (*Lathyrus sativus* L.), an accumulation of free polyamines due to drought stress was correlated with an increase in the β-*N*-oxalyl-l-α,β-diaminopropionic acid (ODAP), and the synthesis of ODAP requires oxalic acid (Xiong *et al.*, 2006). Polyamines have been shown play a large role in environmental stress protection by functioning to reduce

membrane lipid peroxidation, scavenging free radicals, and other mechanisms (Pang *et al.*, 2007). Thus, in *SAG12-ipt* plants, oxalic acid could indirectly play a role in drought tolerance. Malic acid accumulated to a greater extent in *SAG12-ipt* plants. In crested wheatgrass (*Agropyron cristatum*), malic acid was shown to accumulate due to drought stress and this was attributed to protection of roots from drought via accumulation in root exudates (Henry *et al.*, 2007). Specific differences in the content of some of the organic acids could have an effect on the differential drought tolerance characteristics of *SAG12-ipt* and NT plants. It should be noted that differential accumulation of organic acids could also be related to respiratory processes such as glycolysis. Rivero *et al.* (2007) have found photorespiration to be enhanced in *ipt* transgenic tobacco plants and previously several enzymes involved in respiration and energy production, such as glyceraldehyde phosphate dehydrogenase (GAPDH), leaf aldolases, triose phosphate isomerases, and enolases have been found to be maintained better in *SAG12-ipt* bentgrass under drought stress (Merewitz *et al.*, 2011*b*). However, how most of the organic acids relate to the drought response or CK content remains to be determined due to the complexity of the metabolic pathways.

Organic alcohols such as glycerol, myo-inositol, and other organic alcohols are involved in promoting stress tolerance, particularly through osmotic adjustment during salt and drought stresses (Shevleva *et al.*, 1997; Nelson *et al.*, 1998; Bartels and Sunkar, 2005). Several organic alcohols were maintained at greater levels in *SAG12-ipt* plants compared with NT plants under drought stress. Organic alcohols are beneficial osmoregulators that are generally non-toxic to

cells and have a relatively low energy cost of production (Chen and Jiang, 2009). Glycerol-3-phosphate was also greater at equivalent water deficit in *SAG12-ipt* plants compared with NT plants, and is primarily used metabolically for rapid generation of NAD^+ (Shen *et al.*, 2006). Recently, glycerol and glycerol-3-phosphate, in addition to their essential role in glycolysis and lipid formation, have been found to be important components of signalling the defence response against biotic stress (Venugopal *et al.*, 2009). Partra *et al.* (2010) found that transgenic tobacco plants with enhanced contents of myo-inositol and other inositol derivatives exhibit better growth and photosynthetic activity, and a reduction of oxidative stress during salt stress. Previously a promotion of photosynthesis and photosynthetic proteins and reduced oxidative damage and increased antioxidant enzyme activity were found in *SAG12-ipt* plants (Merewitz *et al.*, 2010, 2011*a*, *b*). Therefore, it is worth noting that differences in organic alcohols could be related to various stress-protective mechanisms in addition to osmotic adjustment and signalling as found previously. The importance of organic alcohols in the response of creeping bentgrass to CK and drought stress may be worth investigation.

In conclusion, the greater drought tolerance of creeping bentgrass plants with an *ipt* transgene promoting elevated levels of endogenous CK was associated with changes in the total content of several major metabolites and differential accumulation of some specific metabolites. The most significant metabolites that exhibited greater accumulation in *SAG12-ipt* than in NT plants under drought include amino acids such as alanine, glycine, valine, proline, GABA,

ethanolamine, and pyroglutamic acid, carbohydrates such as fructose, glucose, and galactose, organic acids such as citric acid, malic acid, and oxalic acid, and the organic alcohol myo-inositol and glycerol-3-phosphate. These compounds could be related to stress defence due to their roles in the stress response pathways such as stress signalling, osmotic adjustment, and respiration for energy production. The method of maintenance of these compounds (i.e. due to less degradation or enhanced biosynthesis) and their relationship to CK may deserve further investigation.

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TABLES AND FIGURES

Table 1 Derivitization, relative retention times, and mass to charge ratios used for identification and quantification of the 45 metabolites identified in non-transgenic (NT) and *SAG12-ipt* creeping bentgrass leaves.

Compound	Derivative	Retention time (min)	Mass/charge ratio (m/z)
lactic acid	O,O-TMS*	7.974	219
glycolic acid	O,O-TMS	8.394	205
pyruvic acid	O-TMS, H-TMS	8.654	217
alanine	N, O-TMS	9.047	218
oxalic acid	O, O-TMS	10.021	190
PAME	O,O-TMS	11.127	256
malonic acid	O,O-TMS	11.754	233
valine	N, O-TMS	12.008	246
serine	O,O-TMS	13.181	234
ethanolamine	N, N, O-TMS	13.395	262
H3PO4	O,O,O-TMS	13.582	299
glycerol	O, O,O-TMS	13.682	293
isoleucine	N, O-TMS	14.142	260
threonine	O,O-TMS	14.182	248
proline	N, O-TMS	14.222	216
glycine	N, N, O-TMS	14.448	276
succinic acid	O,O-TMS	14.788	262
glyceric acid	O, O,O-TMS	15.176	307
glycol	O,O-TMS	15.435	191
itaconic acid	O,O-TMS	15.529	259
fumaric Acid	O,O-TMS	15.789	245
methylmaleic acid	O,O-TMS	17.096	259
malic acid	O, O,O-TMS	19.330	335
pyroglutamic acid	N,O, TMS	19.990	258
aspartic acid	N, O, O-TMS	20.070	334
GABA	N, O-TMS	20.237	304
threonic acid	O, O,O-TMS	21.037	409
hexanoic acid	N, O, O-TMS	22.457	363
asparagine	N, N, O-TMS	23.621	348
ribose	MEOX**1, O-4TMS	23.731	307

aconitic acid	O, O,O-TMS	25.492	375
glycerol-3-P	O, O, O,O-TMS	25.626	445
2-KGA	O-5TMS	26.466	437
shikimic acid	O-4TMS	26.740	462
citric Acid	O-4TMS	26.834	465
fructose	MEOX1, O-5TMS	27.896	364
glucose	MEOX1, O-5TMS	28.397	319
galactose	MEOX1, O-5TMS	28.731	319
dopamine	N, N, O, O-TMS	31.866	426
myo-inositol	O-6TMS	32.060	393
tryptophan	N, N, O-TMS	34.379	405
floridoside	O-6TMS	36.178	361
melibiose	O-8TMS	39.343	361
sucrose	O-8TMS	41.466	451
maltose	O-8TMS	59.018	361

Figure 1 Total relative content of the major metabolite groups based on comparison with ribitol as an internal standard and total area of peaks within a given treatment for null transformant (NT) and *SAG12-ipt* transgenic creeping bentgrass leaves in response to drought stress. LSD bars and different letters indicate significant differences at each level of soil water content (% SWC) within a plant type (P ≤ 0.05).
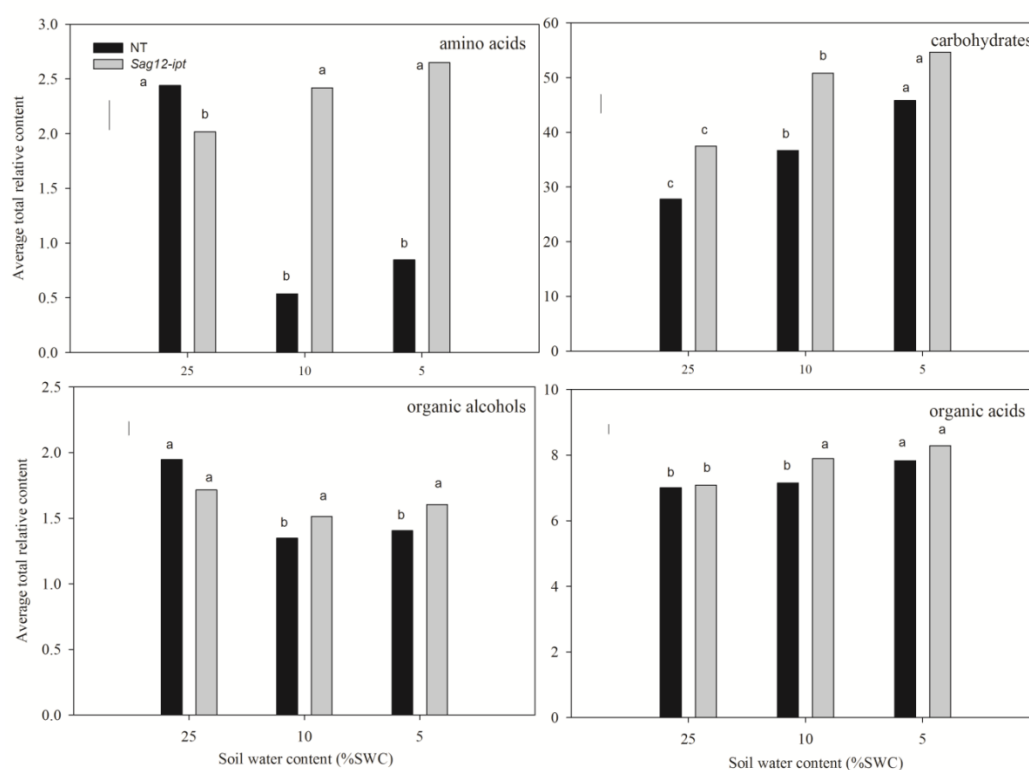
Figure 2 Relative amino acid and amino acid derivative content based on comparison with ribitol as an internal standard and total area of peaks within a given treatment generally (A) increased (B) reduced or (C) alternately accumulated in leaves of null transformant (NT) and *SAG12-ipt* transgenic creeping bentgrass in response to drought stress. Vertical bars indicate stander error values of the means. Letters indicate significant differences due to soil water content (% SWC) within a plant type and asterisks indicate significant differences at a given level of SWC between plant type (P ≤ 0.05). Note the y- axis scaling differences for ease of comparison among treatments of a given metabolite.

Figure 3 Relative carbohydrate content based on comparison with ribitol as an internal standard and total area of peaks within a given treatment generally (A) increased (B) either reduced or alternately accumulated in leaves of null transformant (NT) and *SAG12-ipt* transgenic creeping bentgrass in response to drought stress. Vertical bars indicate stander error values of the means. Letters indicate significant differences due to soil water content (% SWC) within a plant type and asterisks indicate significant differences at a given level of SWC between plant type (P ≤ 0.05). Note the y- axis scaling differences for ease of comparison among treatments of a given metabolite.

Figure 4 Relative content of organic acids based on comparison with ribitol as an internal standard and total area of peaks within a given treatment generally exhibiting an increase in accumulation in leaves of null transformant (NT) and *SAG12-ipt* transgenic creeping bentgrass in response to drought stress. Vertical bars indicate standard error values of the means. Letters indicate significant differences due to soil water content (% SWC) within a plant type and asterisks indicate significant differences at a given level of SWC between plant type (P $\leq$ 0.05). Note the y- axis scaling differences for ease of comparison among treatments of a given metabolite.
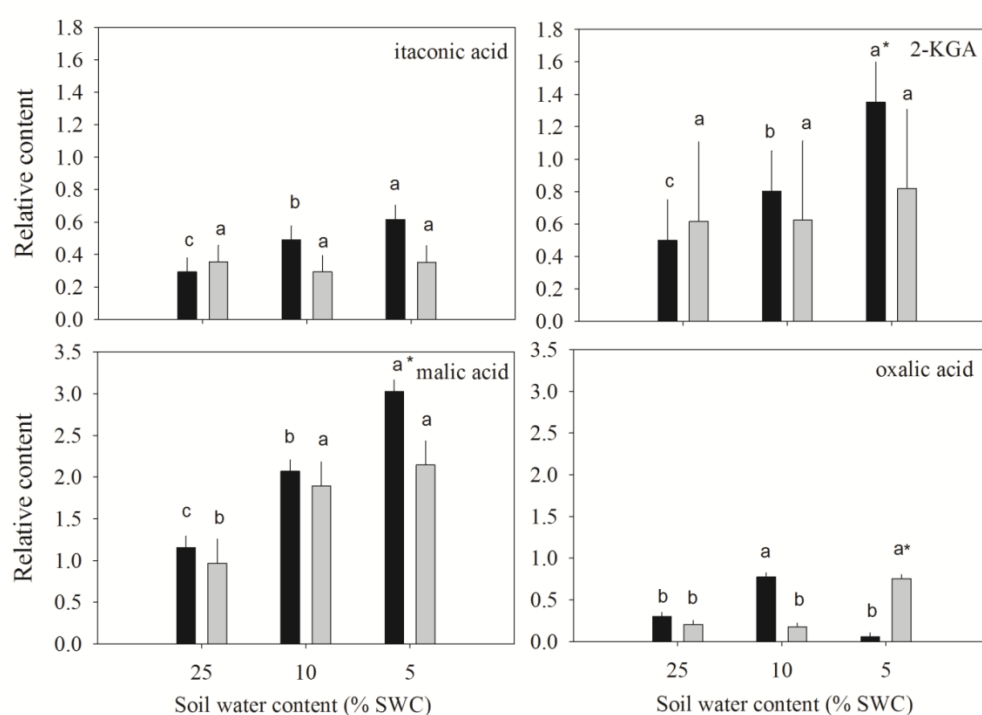
Figure 5 Relative content of organic acids based on comparison with ribitol as an internal standard and total area of peaks within a given treatment generally (A) reduced or (B) alternately accumulated in leaves of null transformant (NT) and *SAG12-ipt* transgenic creeping bentgrass leaves in response to drought stress. Vertical bars indicate stander error values of the means. Letters indicate significant differences due to soil water content (% SWC) within a plant type and asterisks indicate significant differences at a given level of SWC between plant type (P ≤ 0.05). Note the y- axis scaling differences for ease of comparison among treatments of a given metabolite.

Figure 6 Relative content of organic alcohols based on comparison with ribitol as an internal standard and total area of peaks within a given treatment in response to drought stress in leaves of null transformant (NT) and *SAG12-ipt* transgenic creeping bentgrass leaves. Vertical bars indicate stander error values of the means. Letters indicate significant differences due to soil water content (% SWC) within a plant type and asterisks indicate significant differences at a given level of SWC between plant type (P ≤ 0.05). Note the y- axis scaling differences for ease of comparison among treatments of a given metabolite.

Figure 7 Relative content based on comparison with ribitol as an internal standard and total area of peaks within a given treatment expressed as a change in content from the control condition to 47% leaf relative water content (RWC) of null transformant (NT) and *SAG12-ipt* transgenic creeping bentgrass leaves in response to drought stress.
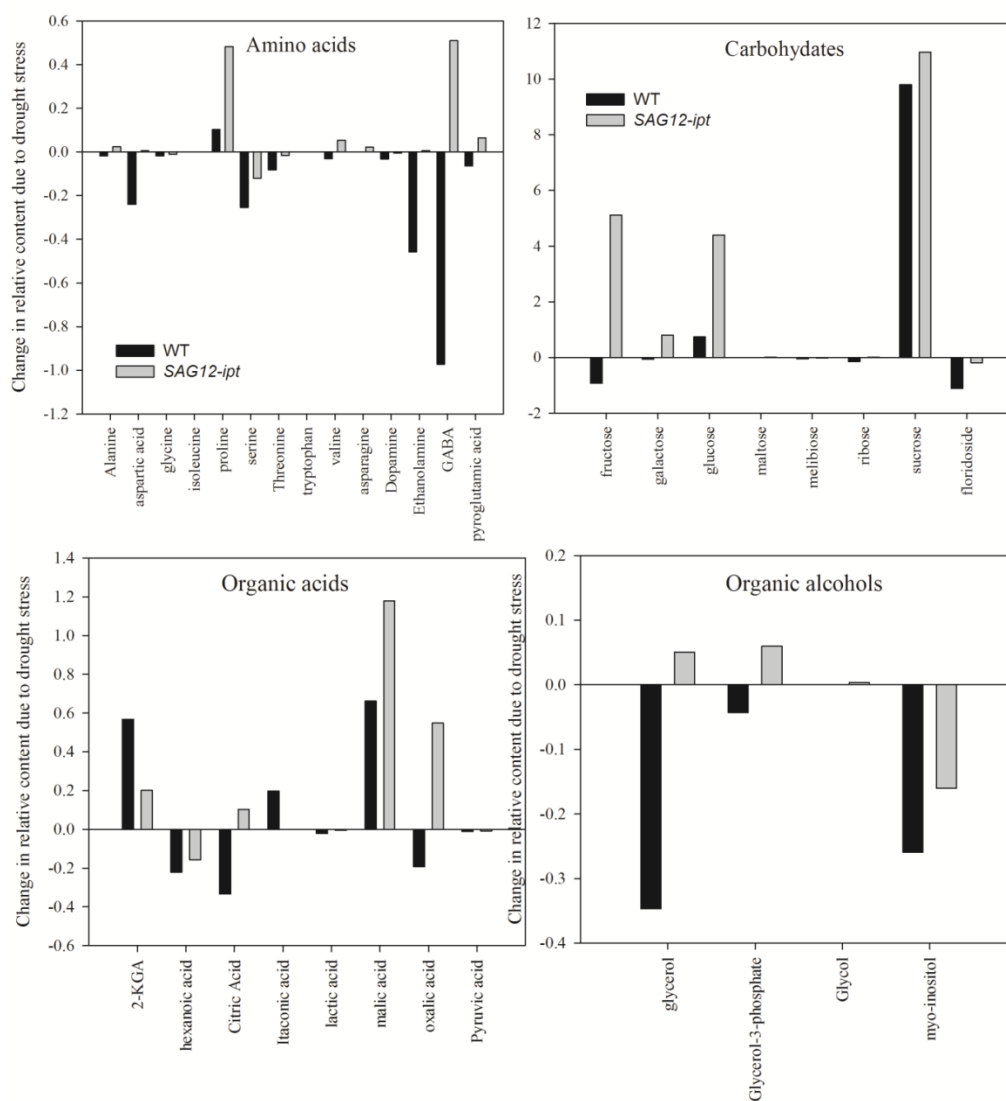
CHAPTER 5

DIFFERENTIAL GENE EXPRESSION IN TRANSGENIC

CREEPING BENTGRASS CONTAINING A GENE FOR CYTOKININ

BIOSYTHESIS DURING DROUGHT STRESS

INTRODUCTION

Creeping bentgrass is an important turfgrass species that is relatively sensitive to drought stress damage. Drought stress symptoms in creeping bentgrass appear after a short period of drought and include wilting, necrotic regions, and reductions in growth rate (Turgeon, 2008). The drought tolerance of an important turfgrass species, creeping bentgrass, has been enhanced by transformation with an *ipt* gene promoting cytokinin (CK) biosynthesis (Merewitz et al., 2010a,b; 2011). The *ipt* transgene codes for the enzyme isopentyl transferase that converts a CK precursor into the active CK, isopentyl adenine. This transgenic system has been shown to increase drought tolerance in various plant species (Rivero *et al*. 2007, Clark *et al*. 2004, Merewitz *et al*., 2010ab, Zhang and Gruissem, 2005). Comprehensive evaluation of the drought response of *SAG12-ipt* transgenic plants compared to the null transformant (NT) and WT plants have been reported previously. *SAG12-ipt* plants exhibit a greater ability to maintain cytokinin content and improvements in major physiological characteristics governing drought tolerance were observed such as reduced electrolyte leakage, maintenance of relative water content, improvements in photosynthetic potential, and reductions in oxidative damage (Merewitz et al., 2010, 2011a,b).

Proteomic evaluation of *SAG12-ipt* creeping bentgrass revealed major changes in the proteome during drought stress compared to NT plants. Protein abundance and activity assays revealed that enhanced CK may elicit improved

physiological characteristics associated with a reduction in leaf senescence and drought damage via maintenance of greater antioxidant enzyme activities (e.g. superoxide dismutase, peroxidases, 2-Cys peroxiredoxin, and catalase). In addition to stress protective proteins, proteins associated with major metabolic pathways such as energy production, carbon and nitrogen metabolism, protein synthesis and destination were generally maintained to a greater extent *SAG12-ipt* plants compared to NT (Merewitz et al., 2011). Evaluation of gene changes that may be consistent with protein changes may indicate targets for CK signaling during drought stress in creeping bentgrass plants. Metabolomic analysis revealed results that were consistent with proteomic results since major metabolites such as carbohydrate, amino acids, and organic acids involved carbohydrate metabolism and energy production pathways accumulated to a greater extent in *SAG12-ipt* plants compared to NT. Gene changes identified here may help in elucidating why protein and metabolite accumulation were differential due to enhanced CK content under drought stress. Much work has been reported on the effects of major hormones involved in the drought response on gene expression, however, less attention is paid to the role of CK in regulating drought responses. For example, Huang et al (2008) detected drought regulated genes that were regulated by hormones other than ABA such as CK in Arabidposis. They found 46 overlapping gene changes common to both drought and CK but were not discussed further.

Subtractive suppressive hybridization (SSH) is known to be an effective way to understand differential responses in gene expression or transcript

accumulation as they relate to different plant types or environmental growth conditions (Hara et al., 1991). Recent advances in SSH kit availabilities and methodologies allow for an exceptional normalization and enrichment for unique sequences after a single subtraction event has removed sequences common to both tester and driver cDNA populations (Diatchenko et al 1996). Utilization of the SSH method has been shown to be a valid way to identify gene expression differences between sensitive and tolerant plant germplasm to different abiotic stresses such as drought. SSH evaluation followed by sequence analysis has revealed the identity of genes that play a role in conferring drought tolerance in a number of crop species such as cotton (Zhang et al., 2009), soybean (Clement et al., 2008), corn (Li et al., 2007) and wheat (Wang et al., 2005). In grass species, SSH has been performed to evaluate Fescue sp. (*Festuca* sp.) in response to heat stress (Zhang et al, 2005). However, relatively little work has been done on expression analysis at the gene level as they relate to drought stress of turfgrasses. Therefore, the objectives of the study were to evaluate gene expression changes due to drought stress and due to the presence of the *SAG12-ipt* transgene causing elevated CK content under both well-watered and drought stressed conditions in creeping bentgrass.

MATERIALS AND METHODS

Plant material

A null transformed line of creeping bentgrass 'Penncross' (NT) and a transgenic line containing the *ipt* gene linked to the *SAG12* promoter (*SAG12-ipt* plants) were exposed to different watering treatments in an environmental growth chamber. The *SAG12-ipt* plants were transformed using the *Agrobacterium* method as described previously in (Merewitz *et al*., 2010; 2011 a, b, c; Xing *et al*., 2010; Xu *et al.*, 2009). *SAG12-ipt* expression caused elevated endogenous CK during drought stress and exhibited physiological characteristics associated with enhanced drought tolerance compared to NT plants in our previous drought stress studies (Merewitz *et al*., 2010) In addition to differential responses of physiological attributes, northern blot analysis, changes in hormone content, and differential regulation of proteomic and metabolomics responses of *SAG12-ipt* lines to drought stress relative to NT have been previously reported (Merewitz *et al*., 2010, 2011 a,b,c). In order for ease of comparison to previous results, the same plant material used for proteomic and metabolomic evaluation (Merewitz et al., 2011 b, c) was used for SSH analysis. Briefly, plants were vegetatively propagated by separating tillers in a greenhouse in Jan 2009. The plants were grown in PVC tubes (40 cm in height x 10.16 cm in diameter) all containing an equal volume of 1:1 fine sand:soil mix (fine-loamy, mixed mesic Typic Hapludult type soil). Plants were watered and fertilized to optimum levels for creeping bentgrass growth in a greenhouse until a full canopy covering the 10.16 cm diameter was achieved. Subsequently, the plants were moved to a controlled-

environment growth chamber in February 2009 (Conviron, Winnipeg, Canada). All plants were fertilized once per week with Hoagland's nutrient solution (Hoagland and Arnon, 1950) and grown in growth chamber conditions consisting of 20/15 °C (day/night) temperatures, 12 h photoperiod, 60% relative humidity, and 500 µmol m^{-2} s^{-1} photosynthetic photon flux density at canopy height. After a 10 d acclimation period, watering treatments were imposed on 3 March 2009.

Watering treatments

Both NT and *SAG12-ipt* plant types were subjected to either well-watered control or water stress conditions (total 80 plants). Replicates of NT and *SAG12-ipt* plants (20 of each) served as the well-watered control plants and were watered once daily to maintain soil volumetric water content (SWC) at approximately 25-30%. SWC was determined with the Time Domain Reflectometry (TDR) method (Topp *et al.*, 1980) using a Trase TDR instrument (Soil Moisture Equipment Corp., Santa Barbara, CA). SWC was measured with one three-pronged waveguide probes (20 cm in length, spaced 2.54 cm apart) installed vertically in each pot, four probes in the control treatment and four probes in the water stress treatment (four replicates in each line). Pot capacity of the soil water was approximately 25%. Water stress was imposed to 20 replicates of each plant type by completely withholding irrigation (40 plants of total). The high number of replicate plants was used to allow for destructive sampling of shoots and roots tissue at 5 different sampling days throughout the experimental study (at 5 levels of drought stress as measured by %SWC and %RWC).

cDNA library construction and SSH analysis

Leaves from the NT and *SAG12-ipt* plants at two levels of cellular water content: well-watered and 47% cellular water deficit (as determined by measurements of leaf relative water content, RWC; Merewitz et al., 2011a) were used to extract total RNA. For total RNA extraction, leaf tissue was ground to a fine powder with mortar and pestle while constantly in the presence of liquid nitrogen. Total RNA was then extracted from the ground leaf tissue with Trizol Reagent, following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The poly adenylated mRNA fraction was isolated from total RNA using an Oligotex isolation midi kit (Qiagen, Valencia, CA, USA). Total RNA content and mRNA concentrations were evaluated using a spectrophotometric method in a NanoDrop instrument (ThermoScientific Co, USA). DNase-treated total RNA (Turbo DNA-free kit, Ambion Inc., Austin, TX) was used for the SMARTer PCR cDNA synthesis kit was used to generate 4 ug of mRNA from each sample to be used in the PCR-Select cDNA subtraction kit (Clontech, Takara BIO, Inc., Mountainview, CA) using the protocols provided by the manufacturer. The PCR-Select cDNA subtraction kit was used to generate both forward and reverse subtraction libraries indicating differential expression between the NT and *SAG12-ipt* plants under both well-watered and drought stressed conditions (47% RWC). A total of 8 cDNA libraries were created in order for performing 8 subtractive hybridizations to generate the subtracted libraries. The treatment expected to contain mRNA of the more drought tolerant or less stressed type (e.g.

well-watered treatment or transgenic line) were utilized as testers in the

hybridizations and the more sensitive were used as drivers (e.g. drought stressed

plants or NT). For instance, in the subtraction comparing both NT and *SAG12-ipt*

under drought stressed conditions, the forward subtraction identified mRNA

transcripts from the drought-stressed *SAG12-ipt* plants was used as the tester and

the mRNA from NT was used as the driver.

Gene cloning, sequence analysis, and experimental design

Plants were arranged as appropriate for a split-plot design with

irrigation treatment as the main plots and plant materials as the sub-plots, with

four replicates for each irrigation treatment and plant material. Sequences

obtained were evaluated and gene identity matches were selected for those that

had a significantly low e value (e value $< 10^{-5}$). Colonies were plated in glycerol

stock and kept at -80C until sequencing. Colonies were selected and sent for

sequencing utilizing M13 reverse primers (Genewiz, South Plainfield, NJ). Gene

sequences were categorized based on their predicted function based on the system

used previously in Bevan *et al*. (1998) and Merewitz *et al.* (2011). Sequences

were evaluated to remove redundant base pairs and vector sequences manually

and through use of VecScreen BLAST database. Clean Blastnr and BLAST of

EST databases were performed (Alschul et al., 1990).

RESULTS

Comparing across hybridization libraries or genes that may have marginally differential gene expression can be difficult due to the potential for gene transcript loss during the experimental procedure and general limitations of sensitivity of the SSH approach. Therefore, specific changes will be discussed and interpreted only as they relate to the respective hybridization. The cDNA library construction and SSH subtraction has successfully revealed 252 gene transcripts that were alternately regulated due to plant type or watering treatment. Of which, a total of 170 clones were selected for sequencing. After removal of redundant sequences and those that were unreadable and BLAST database searches, approximately 136 sequences were successfully identified or were of unknown function in the GenBank database and grouped into functional categories (Figure 1). Differential gene regulation of 18 sequences occurred under well-watered conditions due to the *ipt* transgene and included genes involved in the Metabolism, Energy, Protein synthesis, Disease/stress defense, and unknown categories (Table 1). A total of 52 genes in the forward and reverse libraries comparing NT plants under the well-watered and drought stress condition were successfully sequenced (Table 2). For the *SAG12-ipt* libraries comparing well-watered to drought stress conditions, 26 genes exhibited differential expression (Table 3). The results of the libraries comparing NT and *SAG12-ipt* plants both under drought stress were a total of 40 genes being alternately regulated, including those that were up-regulating and down-regulated (Table 4).

DISCUSSION

Gene expression changes and functional categorization

Under well-watered conditions, gene expression differences could be due to transgene insertion into the genome causing changes in gene expression or due to natural senescence causing the *SAG12* promoter to be activated. No transcripts for the *ipt* gene were directly detected under either well-watered or drought stress conditions, which could be due to the transcript number being too low to detect by SSH. Limitations of the SSH method is that it may preferentially enrich genes with larger initial differences or transcript loss during SSH procedures may occur (Desai et al., 2000; Ji et al., 2002). However, genes that could play a role in hormone biosynthetic pathways were found to be differentially expressed as discussed below. Despite limitations, the results have revealed valuable information regarding alterations in gene expression due to the *ipt* gene during drought stress. Due to the large number of changes, not all genes will be explicitly discussed however some interesting gene changes will be described, particularly if similar results were found in our proteomic and metabolomics studies.

The functional categories having the greatest number of genes differentially expressed in response to both plant type and water treatment in the majority of the libraries were in the Metabolism, Energy, or Unknown categories. Consistent results were found for the differential expression of proteins and metabolites in these plants (Merewitz et al., 2010, 2011a,b). This could be related to the high sensitivity to drought stress of processes that fall within these

categories such as photosynthesis, respiration, and glycolysis as well as the strong regulation effects exhibited by CKs on these and other growth related pathways. The consistency of previous protein and metabolomics work will be used to validate the discussion related to the gene transcript changes. Future confirmations made by RT-PCR analysis will be performed in order for confirmation of these gene expression changes.

Drought stress caused an up-regulation of many genes in both NT and *SAG12-ipt* plants, which is consistent with reports from other plant species such as Arabidopsis (Huang et al., 2008) and rice (Gorlanta, et al 2005). Several genes were found in the NT forward and reverse subtraction libraries but were not found in the *SAG12-ipt* libraries and vice versa. This is due to the differential stress incidence and because a cDNA library is a snapshot of gene expression at a given time point which can be hard to replicate. For instance, few genes (approximately 30) showing differential expression were repeatedly detected in multiple trials of drought treatment in Arabidopsis (Bray, 2004).

The amount of gene overlap identified between the NT and *SAG12-ipt* creeping bentgrass during drought stress was limited. This is a common occurrence in methods evaluating snapshots of gene expression and could be related to different response pathways being active during the different levels of cellular stress. The results suggest that almost twice the number of gene changes occurred due to drought stress in NT plants (52) compared changes in *SAG12-ipt* plants (26) relative to their respective controls. This may reflect less drought

damage to *SAG12-ipt* plants. Fewer gene changes occurring in more drought tolerant plant types compared to sensitive types is a common finding. Hazen et al. (2005) also reported less gene changes in rice plants that had less osmotic adjustment or were more sensitive to drought stress compared to a much larger number of changes in the tolerant plant type exhibiting higher osmotic regulation. For instance, a thioredoxin chloroplast precursor showed greater up-regulation of transcripts in the sensitive type compared to the tolerant type. NT plants generally had greater number of gene changes in the metabolism and disease/stress defense categories whereas *SAG12-ipt* exhibited less. These results may suggest a greater degree of stress damage in NT plants. Zhang et al. (2005) found that the more heat tolerant grass in their study of tall fescue (*Poa pratensis* L.) exhibited maintenance of transcripts related to energy production (particularly photosynthesis) protein synthesis, signaling and transcription factors. Plants that were more sensitive exhibited greater changes in metabolism and stress defense categories.

Metabolism

Gene transcripts that were differentially expressed under well watered conditions due to the *ipt* transgene in the metabolism category had homology to a β-glucosidase gene, SAMS, and a protein phosphatase were detected to be greater in the *SAG12-ipt* plants compared to NT. The glucosidase proteins are a family of enzymes involved in various cellular functions including regulating cellular structure, defense, and in the regulation of plant hormones (Xu et al., 2004). SAMS and protein phosphatase levels were also detected to have lower protein

abundance in NT plants compared to NT plants. The regulation of these proteins

that play a major role in controlling the accumulation of free amino acids or of

those destined for proteogenesis. SAMS activity may be a source of methyl

groups for compounds involved in osmotic adjustment under stress conditions

(Bohnert *et al*., 1996), however how SAMS may be related to CK under non-

stressed conditions is not clear. Auxins have primarily been associated with the

regulation of SAMS content (Gomez-Gomez and Carrasco, 1996).

Under drought stress conditions, transcripts involved in major metabolic

pathways exhibited both down regulation and up-regulation in NT and *SAG12-ipt*

plants, are primarily involved in sugar or nitrogen metabolism. Several gene

expression changes in the metabolism category are consistent with our previous

protein and metabolomics results (Merewitz et al., 2011a,b). For instance, malate

dehydrogenase transcripts and enzymes, and malic acid were all differentially

expressed between NT and *SAG12-ipt* plants. Malate dehydrogenase is a key

enzyme in the citric acid cycle the transcripts were increased by drought stress in

NT plants, but lower accumulation of this protein and malic acid was detected in

NT plants. Therefore, the up-regulation could function to replace damaged

enzymes. Similar results regarding malic acid content have been found in other

plant species (Talame et al 2007). Thiamine biosynthesis protein was found to be

down-regulated by drought stress only in NT plants. Thiamine is used in the

biosynthesis of secondary metabolites such as gamma-aminobutyric acid

(GABA). By metabolite analysis the content of GABA and other secondary

metabolites was maintained to a greater extent in *SAG12-ipt* plants compared to

NT under drought stress, which could be related to this regulation of the thiamine biosynthesis protein. Similarly, the differential regulation of other transcripts in the metabolism category such as glycogen synthase and malate dehydrogenase could be associated with the differential sugar accumulation and glycolytic activities detected between NT and *SAG12-ipt* plants (Merewitz et al., 2011). More detailed studies regarding the effects of CK and drought on respiration would be useful to clarify these differential changes.

Differences in carbon requirements and allocation may be a major factor determining the differential drought response of NT and *SAG12-ipt* plants as they relate to carbon metabolism Drought stress causing an up-regulation of transcripts related to carbon metabolism may be explained by how under drought stress conditions carbon availability is increased due to growth reduction and accumulation of carbon sources (i.e. osmolytes) outweighing photosynthetic limitation (stomatal or non-stomatal) caused by drought damage (Hummel et al. 2010). In addition, there is not always a down-regulation of enzymes or transcripts involved in carbon metabolism since various essential enzymes require replacement. Drought stress has also been shown to increase the expression of genes controlling the central processes of C metabolism. This is due to a need for C mobilization or due to the increased need for replacing damaged enzymes to allow for metabolic processes to remain functional. Increased CK content under drought stress may further protect photosynthetic components and thereby further

amplify carbon availability. The primary source and availability of C of crop plants may be highly affected by reproductive status and time until flowering. These complexities are not an issue in a perennial turfgrass system.

The increase in glycogen synthase kinase (GSK) transcripts in NT plants could be associated with an increased need for sugar for stress defense. In rice plants, an increase in GSK was also exhibited due to drought stress (Wei et al 2006) and may play a role in wound signaling (Jonak et al., 2000). It is unlikely that GSK transcript expression up-regulation would be related to an increase in glycogen synthesis, particularly due to observations in the NT plants of the loss of photosynthetic proteins and reduction in photosystem health allowing for enough sugar production to go into glycogen reserves. GSK may inactivate glycogen synthase or be playing a role in other metabolic functions such as the inactivation or activation of other transcription factors. Xylose isomerase like protein transcripts that were detected to be greater in *SAG12-ipt* transgenic plants compared to NT both under drought stress could be directly related to the expression of the *ipt* transgene, since O-xylosyltransferases are involved in CK hormone biosynthesis (Mok and Mok, 2001). Alternately, xylose isomerases may also be involved in sugar metabolic pathways and are often enhanced due to drought stress (Oono et al., 2003)

Energy production

Changes in genes with function in photosynthetic pathways generally showed an increase or a decrease in transcript expression in both plant types in

response to drought stress. The alternate change in expression may reflect the dynamics between the necessity of maintenance of photosynthetic health of enzymes such as RuBisCo to maintain carbohydrate production and replace damanged enzymes as well as a reduced level of expression due to the slower rates of photosynthesis. RuBisCo transcripts maintained to a greater extent in heat tolerant tall fescue plants (Zhang et al., 2005). Transcripts coding for a Mg-protoporphyrin IX type domain were identified in the forward library of *SAG12-ipt* plants under drought. Based on sequence match results it is not known which enzyme the Mg-protoporphyrin IX domain may be a part of, however, in plants these IX type domains are most commonly a part of the chlorophyll biosynthetic enzyme, Mg-protoporphyrin IX monomethyl ester cyclase. This expression and content of this enzyme is typically reduced by drought stress and maintenance of this enzyme has been associated with improved drought tolerance in transgenic rice (Phung et al., 2011). The *SAG12-ipt* plants maintain chlorophyll content and photosynthetic health to a greater extent than NT plants during stress (Merewitz et al., 2010). Therefore, the increased in expression of these transcripts may reflect differences in chlorophyll maintenance in plants with elevated CK content. Interestingly, the CAT is known to stimulate the activity of this type of protoporphyrin cyclase (Bollivar and Beale, 1996). We have found CAT to potentially be a primary factor related to enhanced CK content in the *ipt* plants under drought stress, as discussed below. Transcripts involved in respiration pathways such as GAPDH were down-regulated in NT plants. A corresponding decrease in abundance of GAPDH proteins and intermediates in respiratory

pathways also occurs in response to drought in NT plants (Merewitz et al.,

2011a). Therefore, *SAG12-ipt* plants may be better able to maintain the health of

respiratory pathways compared to NT plants.

Transporters

A few types of transporters were found to be differentially regulated by

drought stress in *SAG12-ipt* plants due to drought stress or were greater in the

SAG12-ipt line compared to NT plants under stress. An aquaporin Pip1-2, a type

of water transporting channel, was up-regulated by drought stress in *SAG12-ipt*

plants. In chickpea, a greater and more rapid expression of aquaporin transcripts

occurred in drought tolerant lines compared to a drought sensitive cultivar (Jain

and Chattopadhyay, 2010). The up-regulation of aquaporins during drought stress

has been reported in other crop species such as Arabidopsis (Seki et al, 2002) and

rice (Gorlanta, et al., 2007). In addition to water movement, aquaporins also play

an important role in mobilizing other metabolites throughout the plant, including

those of small molecular weight such as glycerol and urea and gases such as NH_3

and CO_2 (Maurel, 2007). Metabolites that were important in the drought response

in *SAG12-ipt* plants included several small molecular weight compounds

including glycerol. The up-regulation of these transports may play an important

role in allowing water and metabolite movement during stress periods.

Interestingly, CK and the hormone CK:ABA ratio is known to regulate turgor

pressure via aquaporins (Chernyad'ev, 2009). It is still unknown how the

increased CK in the transgenic plants does not greatly affect stomatal conductance

or water loss, as may be expected, but the maintenance of RWC in *SAG12-ipt*

plants could be related to this type of differential aquaporin or transporter

expression. Another type of transporter, an ABC transporter was found to be up-

regulated in NT plants. ABC transporter transcripts were greater in tall fescue

sensitive to drought stress (Zhang et al., 2005). Other transcripts that were

increased by drought stress in NT plants but not in *SAG12-ipt* plants include one

coding for an CDGSH iron sulfur domain 1 protein and a vacuolar ATPase.

CDGSH iron sulfur domains are typically located in mitochondrial membranes,

serving as transport channels for electron gradient regulation and iron transport

(Lin et al., 2011). Vacoular ATPases are primarily involved in transporting ions

across the plasma membranes such as Ca^{2+} ions (Sze, 1984). The requirement of

up-regulation of these transcripts could be related to osmotic balance maintenance

requirements or osmotic adjustment in NT plants.

Transcription

Relatively few transcription factors were differentially regulated, which

could be due to the fact that these factors were in common and subtracted out

during hybridization or the plants were experiencing severe drought stress

conditions and transcription factor responses are the first responders to signal

plant responses (Bray, 2004). Differences in gene regulation are highly

conditional upon the duration of stress incidence and tissue sampling time during

drought stress (Ouyan et al., 2007). In addition, the low quanitity of these

transcripts makes detection by SSH more difficult (Desai et al., 2000). Elongation

factor-1-α transcripts were expressed to a greater extent in the tolerant chickpea compared to the sensitive cultivar (Jain and Chattopadhyay, 2010).

Protein destination/storage

Transcripts coding for proteins related to protein transport or protein degradation were the main types of transcripts differentially regulated in the protein destination/storage category and more changes in these transcripts were detected in NT plants than in *SAG12-ipt*. For example, an ATP-dependent Clp protease ATP-binding subunit clpA and transcripts associated with ubiquitin were found to be either up- or down-regulated in NT plants. The ATP-dependent Clp protease ATP-binding subunit clpA homolog may interact with a clpP-like protease involved in degradation of denatured proteins in the chloroplast (reference). Ubiquitin pathways mark proteins for degradation by the proteasome or can function to regulate membrane bound proteasome associated signaling transduction caused by CK during cell division processes (Kim and Park, 2007). Transcripts coding for an ubiquitin-conjugating protein was also found to be down-regulated due to drought stress in barley (Talame et al 2007). Tian et al (2009) also found the ubiquitin proteasome pathway to be up-regulated under heat stress conditions in a grass species with superior heat tolerance. Alternate mechanisms could be employed in regard to protein degradation and stress tolerance since increased accumulation of proteases is important for stress tolerance to facilitate utilization of nutrients in protein turnover whereas a

reduction of protease activity could allow for maintenance of protein health and functionality under stress. Transcripts such as GTPase SAR1 is necessary for initiation of the process of transporting proteins from the exit sites of the Golgi apparatus (Aridor et al., 2001). This could reflect the requirement of newly synthesized proteins to replace those damaged by drought stress.

Signal transduction

Leu-rich repeat (LRR) receptor kinases were decreased in response to drought stress in NT plants but exhibited enhanced expression in *SAG12-ipt*. LRR receptor kinases are typically localized to plasma membranes and are up-regulated by increased ABA content (Osakabe et al., 2005). Interestingly, LRR receptor kinases function are also closely tied to regulation by peptide plant hormones (e.g.phytosulfokine) that are less well understood (Matsubayashi et al., 2002). Differential expression in NT and *SAG12-ipt* plants could be due to variances in hormone content such as the ratio of CK:ABA.

Disease/Stress defense

CAT expression was greater in *SAG12-ipt* plants under the well-watered condition compared to NT plants. CAT is an antioxidant enzyme that detoxifies hydrogen peroxide that can accumulate in plant cells and is an essential enzyme for stress tolerance. Tobacco plants deficient in the CAT enzyme were found to be greatly susceptible to abiotic stress (Willekens et al., 1997). Enhancement of the expression and activity of CAT promotes stress tolerance (Franca et al., 2005). CAT expression in *ipt* transgenic tobacco was enhanced by drought stress to a

greater extent than in non-transgenic plants (Rivero *et al*., 2007).Several grass species consistently show stable expression of CAT under drought stress (Bian and Jiang, 2009; Jiang et al., 2010; Merewitz et al., 2011). In addition, leaf senescence is correlated with a decline in CAT activity (Dhindsa et al., 1981) and CKs have been shown to increase CAT activity (Zavaleta-Mancera et al., 2007). Therefore, the prevention of natural leaf senescence in the well-watered condition and the maintenance of protein content and activity of CAT under drought stress (Merewitz et al., 2011) may be a main factor promoting greater drought stress tolerance in *SAG12-ipt* transgenic creeping bentgrass with enhanced CK content. The stability of CAT during drought stress and its great capacity to reduce cellular stress in grass species may make this a highly effective and valuable molecular target for enhancing or selecting drought tolerant grass germplasm in breeding programs.

Glyoxalase is another stress protective enzyme that accumulates in response to drought conditions in order to break down a toxic by-product of glycolysis, methyl-glyoxyl. In the drought tolerant resurrection grass (*Sporobolus stapfianus*), glyoxylase transcript expression were also up-regulated in response to drought (Gaff et al., 1998). The accumulation of glycolytic proteins (Merewitz et al., 2011) and transcripts involved in respiration detected in *Sag12-ipt* plants such as GAPDH compared to NT, may be related to an increased flux through glycolysis and increased need for the glyoxalase enzyme. In addition, metabolite analysis revealed the accumulation of intermediates in the glycolysis pathway to a greater extent in Sag12-ipt plants than in NT plants during drought stress

(Merewitz et al., 2011). A rapid stimulation of glycolysis is thought to be a beneficial early drought stress tolerance mechanism, as also evident in another resurrection plant *Craterostigma plantagineum* (Velasco, et al., 1994), for readily available free sugars for osmotic adjustment and energy production. This increased flux through glycolysis may play a role in CK regulation of drought tolerance.

A universal stress protein (USP) was detected in NT plants due to drought stress. An accumulation of the USP 1 transcripts was also detected due to drought stress in rice (Hazen et al., 2005) and is responsive to other abiotic stress such as cold (Kosmala et al 2009). USPs have been implicated to be factors that control differential cultivar variation in stress tolerance. Different studies have found contrasting results regarding the expression or accumulation of USP proteins as they relate to drought sensitivity or tolerance. (Kosmala et al., 2009; Hazen et al. 2005, Li et al., 2010) Species and stress duration and differences in USP isoforms may be the cause of whether an up-regulation or a down-regulation of gene transcripts and differences in USP protein accumulation are correlated with stress tolerance. Similar to our results, various USP genes were more highly expressed in the salt sensitive variety compared to the tolerant type (Li et al., 2010). USP transcripts and protein generally has been associated with stress incidence and accumulates in the cytoplasm when cells are undergoing stress damage (Isokephi, et al., 2011; Harb et al., 2010). Therefore, the NT plants may be up-regulating the USP protein due to cellular damage. Genetic manipulation of USP proteins may be a viable method to improve plant stress tolerance (Isokephi, et al., 2011) How

CK may regulate USP proteins is not conclusive from our *ipt* plant studies. However, since the response occurred in the more drought sensitive NT grass, further investigation into the responses of USPs in turfgrass species may be warranted and enhancement of the response of USP under drought stress in grass species may help promote drought tolerance.

Chloroplast localized ToxA binding protein (Pr ToxA) was detected in the forward library of NT to *SAG12-ipt* under drought stressed conditions, meaning an greater number of Pr ToxA were in *SAG12-ipt* plants. Ptr ToxA function involves ToxA are also differentially responsive to cold in frost tolerant and sensitive *Festuca* grass plants and has been found to be required for PSII stability in chloroplasts (Kosmala, et al., 2009; Manning et al., 2007).How Pr ToxA may be related to drought tolerance is not well known. Perhaps, the consistent promotion of PSII health and maintenance of accumulation of photosynthetic compounds in *SAG12-ipt* plants coupled with the increased Pr Tox transcripts under drought stress could be associated with the CK gene regulatory network.

Gene transcripts with sequences likely coding for jasmonate induced proteins were found to be increased in both NT and *SAG12-ipt* plants in response to drought stress. Similar results were found in drought stressed barley (Talame et al., 2007; Ozturk et al., 2002). Jasmonate and the jasmonic acid hormone signaling pathway are known to be involved in regulation of plant responses to various abiotic stresses and play a role in leaf senescence (Pauwels, et al., 2009). Jasmonic acid induces protective mechanisms such as osmotic adjustment and antioxidant defences in plants and reduced lipid peroxidation in soybean plants

exposed to drought stress (Anjum et al., 2011). Therefore, the up-regulation of these genes in creeping bentgrass could be related to drought stress defense, but our results do not indication that these genes may be differentially regulated by CK.

A transcript coding for a DELLA protein was reduced by drought stress in *SAG12-ipt* plants. Degradation of DELLA proteins is known to alleviate growth repression. DELLA proteins are components of regulatory pathways that are closely associated with plant hormones such as ethylene (Neumann, 2008). Leaf biomass accumulation under drought stress was doubled in plants containing unfunctional DELLA proteins caused by knock-out mutation (Achard, 2006). Therefore, the reduction of leaf senescence due to CK could and the maintenance of metabolic activities in *SAG12-ipt* plants could be related to this differential gene expression.

Secondary metabolism

Transcripts coding for an isoflavone reductase-like protein 5 were found to be greater in *SAG12-ipt* plants compared NT under drought stress. Isoflavone reductase proteins are known to decrease due to drought stress (Salekdeh et al., 2002). Maintenance of greater levels of isoflavone reductase enzymes is associated with drought tolerance (Watkinson et al., 2003). Our results are consistent with those found in loblolly pine, where isoflavone transcripts were up-regulated in response to mild drought stress but not in the severe stress state (Heath et al., 2002). Thus, this suggests less stress damage in *SAG12-ipt* plants at

the same level of cellular water deficit than in NT plants, which is consistent with
our physiological results (Merewitz et al., 2011). Transcripts encoding
iosoflavone reductases were also found to exhibit differential regulation in SSH
libraries constructed comparing differential tall fescue cultivars in response to
heat stress (Zhang et al., 2005). Transcripts coding for a putative dipthine
synthase enzyme were down-regulated by drought stress only in NT plants.

In conclusion, valuable information regarding gene changes associated
with drought stress in creeping bentgrass and how they may relate to enhanced
CK content and drought tolerance has been identified. Genes of particular interest
in how CK may enhance the drought tolerance response were primarily coding for
proteins associated with major metabolic functions such as those regulating
energy production, metabolism, and stress defense. Further studies evaluating the
downstream effects of differentially expressed genes discussed here that were
associated with CK maintenance under drought stress in creeping bentgrass may
be beneficial for further understanding of how these genes may relate to CK
regulation under drought stress in creeping bentgrass.

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TABLES AND FIGURES

Table 1. Genes categorized by protein function based on BLAST searches that were differentially expressed in SSH library A (NT compared to *SAG12-ipt* transgenic plants both growing under well-watered conditions) in the forward (up-regulated) and reverse (down-regulated) libraries. Numbers in parenthesis indicate number of transcript copies detected.

Library	Description	Accession #	E-value
Category 01 Metabolism			
Forward	β-glucosidase	ACF22735.1	6.00E-40
Category 02 Energy			
Forward	enolase (2-phosphoglycerate dehydratase) (2)	AAM69295.1	3.00E-60
	photosystem I P700 chlorophyll a apoprotein A2	ABG66207.1	1.00E-71
	photosystem II precursor, chloroplast	NP_001134061.1	9.00E-68
	RuBisCO large subunit (4)	ADU18941.1	4.00E-98
Reverse	GAPDH	ACV86034.1	2.00E-133
Category 05 Protein synthesis			
Forward	elongation factor 1-alpha-like protein	ABB16977.1	2.00E-79
	nonribosomal peptide synthetase	CBJ23772.1	1.00E-10
	40S ribosomal protein S21	NP_001105477.1	1.00E-47
Category 10 Signal transduction			
Forward	protein phosphatase 1 regulatory subunit 12b	XP_001662854.1	4.00E-18
Category 11 Disease/Stress defense			
Forward	catalase 2	A55092	1.00E-08
	catalase	Q59296.1	3.00E-10
Category 20 Secondary metabolism			
	S-adenosylmethionine synthase 4 (SAMS)	Q4LB21.1	2.00E-60
Category 12 Unclear			9.00E-104
Forward	predicted protein	BAJ87310.1	4.00E-117
	predicted protein	BAJ96581.1	3.00E-36
	predicted protein	BAK02049.1	5.00E-58
	hypothetical protein SORBIDRAFT_01g032220	XP_002467681.1	5.00E-58
Reverse	predicted protein	BAJ93277.1	4.00E-16
	predicted: similar to CG18041 CG18041-PA	XP_969418.1	4.00E-18

Table 2. Genes categorized by protein function based on BLAST searches that were differentially expressed in SSH library B (NT under well-watered conditions compared to WT under drought stressed conditions) in the forward (up-regulated by drought) and reverse (down-regulated by drought) libraries. Numbers in parenthesis indicate number of transcript copies detected.

Library	Description	Accession #	E-value
Category 01 Metabolism			
Forward	malate dehydrogenase	XP_001659012.1	2.00E-29
	glycogen synthase kinase-3 MsK-3	NP_001148880.1	6.00E-07
Reverse	nitrilase-associated protein, putative	CAJ38376.1	4.00E-12
	thiamine biosythesis protein ThiC, putative	AAG49550.1	1.00E-54
Category 02 Energy			
Forward	RuBisCO large subunit (7)	AEK34569.1	2.00E-44
	RuBisCO small subunit 1B	NP_198659.1	0.00001
	RuBisCO small chain c	ABR26034.1	0.00013
	Oxygen-evolving enhancer protein 3-1, chloroplast precursor, putative	BAC83128.1	1.00E-37
	cytochrome c oxidase subunit III	YP_003734710.1	8.00E-36
	Photosystem II 10 kDa polypeptide, chloroplast	NP_001134061.1	4.00E-60
	thioredoxin-like 5, chloroplastic	ABR26107.1	3.00E-30
	ATP-citrate synthase, putative	XP_002519229.1	5.00E-49
Reverse	RuBisCO large subunit	ADU18941.1	2.00E-76
	RuBisCO (2)	CBF07493.1	1.00E-26
	glyceraldehyde-3-phosphate dehydrogenase 1	ACV86034.1	8.00E-107
	oxygen-evolving enhancer protein 1 , chloroplast	ABQ52657.1	9.00E-20
Category 05: Protein synthesis			
Forward	elongation factor 1 alpha (2)	AEG78681.1	2.00E-112
Category 06: Protein destination/storage			
Forward	E3 ubiquitin-protein ligase UBR5	EFN82877.1	2.00E-57
	E3 ubiquitin-protein ligase BRE1-like 1	NP_182022.2	7.00E-05
	GTPase SAR1	ACD03831.1	3.00E-57
	thiol disulfide interchange protein txlA	NP_001152226.1	2.00E-06
Reverse	ubiquitin-60S ribosomal protein L40-like	XP_003465244.1	1.00E-12
	ubiquitin-conjugating enzyme E2-like protein	ADB28900.1	2.00E-14
	ATP-dependent Clp protease ATP-binding subunit clpA, chloroplastic	P31542.1	9.00E-34

Category 07 Transporters

Forward	vacuolar H+-ATPase subunit B	BAF38479.1	2.00E-61
	ABC transporter integral membrane protein	ZP_07303354.1	0.00061
	CDGSH iron sulfur domain 1	NP_001004811.1	

Category 10 Signal transduction

Forward	uridine kinase	ZP_00781761.1	0.00042
	serine/threonine kinase receptor precursor-like protein	BAC57306.1	4.00E-39
Reverse	LRR receptor-like kinase (3)	ACY30448.1	7.00E-70

Category 11 Disease/Stress defense

Forward	jasmonate-induced protein, putative	ABA96835.1	3.00E-08
	universal stress protein 5327	ADB54812.1	4.00E-37
	abscisic acid-responsive HVA22 family protein	XP_002865508.1	3.00E-08
	glyoxalase I	AAW68026.1	1.00E-53

Category 20 Secondary metabolism

Reverse	diphthine synthase, predicted	XP_001604120.1	6.00E-75

Category 12 Unclear

Forward	predicted protein	BAK02049.1	1.00E-23
	predicted protein	BAJ85089.1	3.00E-42
	predicted protein	BAJ96581.1	3.00E-21
	hypothetical protein SORBIDRAFT_10g001310	XP_002436374.1	0.0002
	hypothetical protein pBMB0558_00760	YP_004169259.1	5.00E-04
	predicted protein	BAJ95019.1	1.00E-32
	predicted protein	BAJ99280.1	5.00E-12
	hypothetical protein MELLADRAFT_95019	EGF98952.1	1.00E-23
	hypothetical protein pBMB0558_00760	YP_004169259.1	3.00E-04
	predicted protein	BAJ90401.1	3.00E-16
	hypothetical protein OsJ_26652	EAZ42091.1	6.00E-17
Reverse	predicted protein	XP_001770883.1	0.0004
	hypothetical protein isoform 1	XP_002272271.1	3.00E-08
	hypothetical protein SORBIDRAFT_01g040990	XP_002465552.1	0.00017
	hypothetical protein OsJ_19720	EEE64863.1	1.00E-57
	hypothetical protein Tc00.1047053508475.20	XP_804280.1	0.000031
	hypothetical protein LOC100273563	NP_001141453.1	8.00E-12

Table 3. Genes categorized by protein function based on BLAST searches that were differentially expressed in SSH library C (*Sag12-ipt* under well-watered conditions compared to *Sag12-ipt* under drought stressed conditions) in the forward (up-regulated by drought) and reverse (down-regulated by drought) libraries. Numbers in parenthesis indicate number of transcript copies detected.

Library	Description	Accession	E value
Category 02 Energy			
Forward	Mg-protoporphyrin IX	CAB58179.1	2.00E-60
	RuBisCo large subunit (2)	ADU18941.1	3.00E-76
	Oxygen-evolving enhancer protein 3-1, chloroplast precursor (OEE3)	BAC83128.1	4.00E-76
	Thioredoxin-like 5, chloroplastic	ABR26107.1	
Reverse	Fructose-bisphosphate aldolase, class I	AT3G52930	1.00E-109
	Photosystem II 10 kDa polypeptide, chloroplast	NP_001134061.1	2.00E-17
	Cytochrome c oxidase subunit III	ADO60570.1	2.00E-15
	RuBisCo large subunit	ACO35581.1	2.00E-56
Category 03 Cell growth/division			
Reverse	tubulin α-3 chain	NP_001167663.1	2.00E-58
Category 06 Protein destination/storage			
Reverse	ubiquitin-conjugating enzyme	ADX86831.1	7.00E-51
Category 10 Signal transduction			
Forward	receptor expression-enhancing protein 3 HVA22-like protein	NP_001148675.1	7.00E-31
	LRR receptor-like kinase	ACY30448.1	5.00E-40
	uridine kinase	ZP_00781761.1	6.00E-30
Reverse	Os01g0629400 (phosphatase-like)	NM_001050175.2	8.00E-60
	ctd-phosphatase-like protein	ABR26130.1	8.00E-60
Category 11 Stress/disease defense			
Forward	glyoxalase I (3)	AAW68026.1	4.00E-32
	jasmonate-induced protein, putative	ABA96835.1	5.00E-30
Reverse	DELLA protein RGL1	EG429076.1	0.00E+00
Category 20 Secondary metabolism			
Forward	spermidine synthase	AEL33692.1	1.00E-109
Category 12 Unclear			
Forward	hypothetical protein NCLIV_068840	CCA30004.1	5.00E-31
	hypothetical protein SORBIDRAFT_06g021780	XP_002448130.1	0.011

	OSJNBa0014K14.7	CAE02935.3	2.00E-91
Reverse	hypothetical protein	CAM36311.1	2.00E-08
	predicted protein	BAJ90401.1	5.00E-16

Table 4. Genes categorized by protein function based on BLAST searches that were differentially expressed in SSH library D, which compared NT to *Sag12-ipt* under drought stressed conditions equal in cellular water deficit (47% RWC) in the forward (up-regulated by transgene during drought stress) and reverse (down-regulated by transgene during drought stress) libraries. Numbers in parenthesis indicate number of transcript copies detected.

Library	Description	Accession	E value
Category 01 Metabolism			
Forward	GDP-mannose 3,5-epimerase (2)	XM_003577361.1	3.00E-27
	Probable mannose-1-phosphate guanylyltransferase 3	Q6Z9A3.1	3.00E-149
	mannose-1-phosphate guanylyltransferase	NP_181507.1	1.00E-15
	glycosyl hydrolase family 19 protein	AAQ84319.1	6.00E-44
	UTP--glucose-1-phosphate uridylyltransferase	Q43772.1	1.00E-15
	UDP-glucose dehydrogenase	AAX08057.1	7.00E-25
	xylose isomerase-like	XM_003562448.1	4.00E-23
	xylose isomerase	CAA64544.1	3.00E-25
Reverse	glycine decarboxylase P subunit	AAB82711.1	8.00E-12
	acetyl-CoA carboxylase	NP_001185143.1	4.00E-21
Category 02 Energy			
Forward	RuBisCo large subunit (3)	AAQ08331.1	5.00E-61
	chloroplast-localized Ptr ToxA-binding protein1	AAR24582.1	6.00E-54
Reverse	RuBisCo large subunit (4)	ACO35581.1	4.00E-46
	thioredoxin-like 5, chloroplastic	ABR26107.1	3.00E-30
	ATP-citrate synthase, putative	XP_002519229.1	5.00E-49
Category 04 Transcription			
Forward	partial 16S rRNA gene	FN421445.1	
	histone H3	XP_001752178.1	1.00E-121
	translational initiation factor eIF1	BAF63490.1	2.00E-11
Category 05 Protein synthesis			
Forward	α-1,4-glucan-protein synthase, putative	ABF97477.1	1.00E-30
	Os07g0609766 (Armadillo-like helical	NP_001175295.1	3.00E-05

domain containing protein)

Category 06 Protein destination/storage

Reverse	proteasome subunit beta type-7-B-like	XP_003568783.1	

Category 07 Transporters

Forward	aquaporin PIP1-2	NP_001078067.1	1.00E-32
	coatomer subunit beta'-2 (2)	NP_175645.1	1.00E-49
Reverse	plasma membrane H+-ATPase	CAC50884.1	1.00E-28

Category 10 Signal transduction

Forward	receptor kinase ORK14	AAM09948.1	5.8

Category 11 Disease/Stress defense

Forward	Chloroplast Ptr ToxA-binding protein	AK332987.1	1.00E-45
	glyoxalase I	BAB71741.1	9.00E-26

Category 20 Secondary metabolism

Forward	cruciferin cru4 subunit	X57848.1	
	isoflavone reductase	ACH72670.1	4.00E-57

Category 12 Unclear

Forward	Os11g0169100	NP_001065849.1	7.00E-50
	hypothetical protein [Zea mays]	ACG32534.1	5.00E-13
	predicted protein [Hordeum vulgare subsp. vulgare]	BAK07943.1	1.00E-41
	predicted protein [Hordeum vulgare subsp. vulgare]	BAK05471.1	5.00E-06
Reverse	hypothetical protein [Thermobia domestica]	CAM36311.1	8.00E-07

Figure 1 Percent total changes of gene transcripts (including forward and reverse libraries) in functional categories comparing A) NT to *SAG12-ipt* under well-watered conditions B) NT watered to NT drought stress C) *SAG12-ipt* well-watered to *SAG12-ipt* drought stress D) NT drought to *SAG12-ipt* drought stress conditions
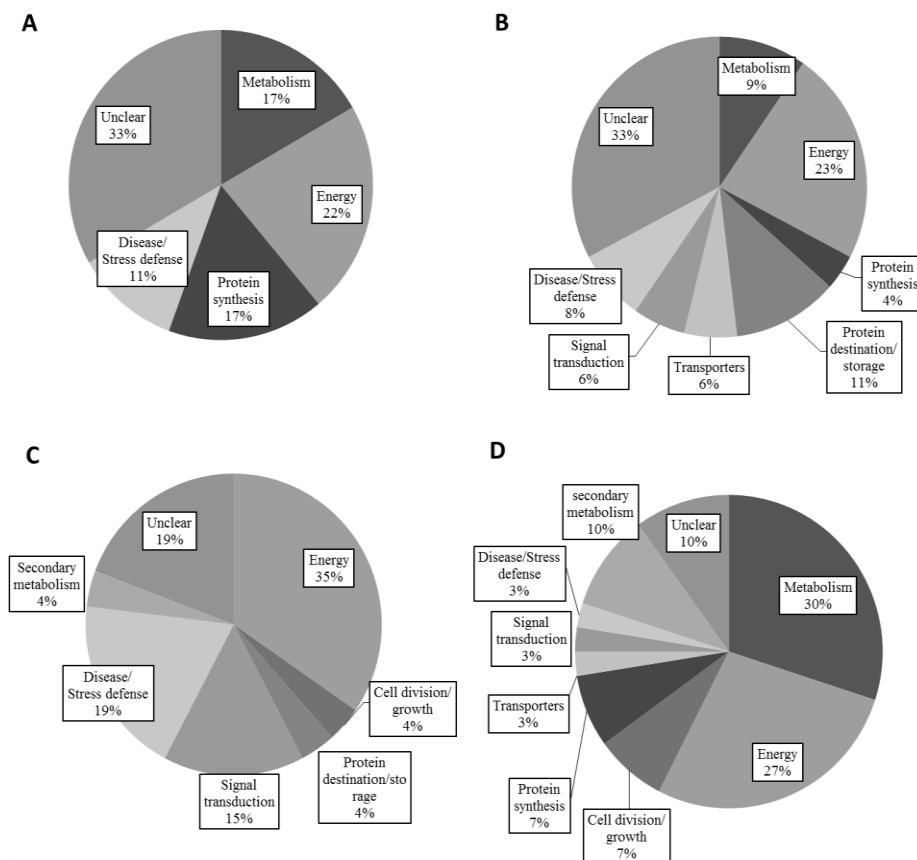
PART II – IDENTIFICATION OF GENOMIC REGIONS
ASSOCIATED WITH DROUGHT TOLERANCE IN TWO
AGROSTIS TURFGRASS SPECIES

CHAPTER 6

IDENTIFICATION OF QUANTITATIVE TRAIT LOCI (QTL)

THAT INFLUENCE DROUGHT TOLERANCE IN A COLONIAL X

CREEPING BENTGRASS HYBRID POPULATION

INTRODUCTION

The identification of quantitative trait loci (QTL) is a valuable method to detect important genomic regions controlling drought stress tolerance traits in plant species. Drought stress is the foremost abiotic stress that limits the growth and productivity of many plant species. Identification of QTLs for various drought tolerance traits has been achieved in several major crop species such as sorghum (*Sorghum bicolor* L.) (Xu et al., 2000), corn (*Zea mays* L.) (Ribaut et al., 1997) (Hao et al., 2010), wheat (*Triticum aestivum* L.) *(*Dashti et al., 2007), and rice (*Oryza sativa* L.,) (Price et al., 2002), as well as model species such as Arabidopsis (*Arabidopsis thaliana* L.) (Juenger et al., 2005). In comparison to annual crops, relatively little information is available regarding genomic information or QTLs for drought tolerance traits in grass species, particularly those used as turfgrass (Fei, 2008). In turfgrasses, QTLs have mainly been identified for prevalent biotic diseases such as dollar spot in creeping bentgrass (Bonos, 2006; Chakraborty et al., 2006) and for gray leaf spot (Jo and Jung, 2006; Curley et al., 2008) and crown rust resistance in perennial ryegrass (*Lolium perenne* L.) (Sim et al., 2007). QTLs for plant morphology (Yamada et al., 2005) and cold hardiness (Xiong et al. 2007) also have been evaluated in perennial ryegrass. However, there is little information regarding QTLs for drought stress tolerance mechanisms in turfgrass species. QTL markers such as these have a great potential to be utilized in breeding improvement for disease resistance.

Evaluating phenotypic traits, which may indicate whole plant responses to drought stress, is important for identifying drought tolerant grasses and will allow for a greater understanding of the genetic control of quantitative traits. Once identified, QTLs for important drought stress tolerance traits can be used in marker assisted selection, for identifying specific genes underlying the QTLs, or for analysis of genomic synteny with related plant species (Edwards et al., 1987). Recent successes in fine mapping have allowed QTL detection and genomic selection utilizing markers to be considered the future of plant breeding (Fei, 2008; Ren et al., 2009; Yang et al., 2010; Lorenz et al., 2011; Salunkhe et al., 2011). QTLs may be used in breeding programs to facilitate and alleviate the complexities of finding grass germplasm that has both drought tolerance characteristics and other complex phenotypic characteristics such as good turf density, color, and spreading traits. For example, the complexity of finding useful QTLs in corn (*Zea mays* L.) not only lies in the complexity of drought tolerance traits themselves, but also QTLs that are associated with genetic regions producing good yield characteristics (Hao et al., 2010). Often these other phenotypic traits such as yield in corn or quality in turfgrasses may not typically be concomitant with drought tolerance traits since some may be considered a trade-off in the plant's natural defenses. For instance, reduced rates or cessation of lateral grass growth are drought defense mechanisms exhibited by grasses, however, these are not desirable phenotypic qualities in the turfgrass industry since there is a desire for functionality as a utility surface and aesthetics.

The objectives of the study were to identify QTLs for both drought resistance characteristics and quality characteristics under watered and drought stress conditions. Germplasm from a colonial x creeping bentgrass hybrid backcross population was evaluated for physiological traits of known importance in drought tolerance, including turf quality for overall turf performance, relative water content, canopy temperature depression, osmotic adjustment, cell membrane stability estimated as electrolyte leakage (EL), chlorophyll content, and the normalized difference vegetative index (Price et al., 2002; Bonos and Huang, 2006). Screening hybrid backcross populations has been found to be an effective method for observing transgressive segregation and locating QTLs due to the phenotypic diversity inherent to such populations (deVicente and Tanksley, 1993). QTLs for drought resistance and quality characteristics reported here could facilitate the development of high quality, drought-tolerant grass cultivars adapted to water-limiting environments.

MATERIALS AND METHODS

Plant Material and Growth Conditions

A backcross population produced from an interspecific hybrid of colonial bentgrass (*A. capillaris* L.) and creeping bentgrass (*Agrostis stolonifera* L.) was phenotypically evaluated for drought tolerance traits. The population and linkage map was originally developed and described previously by Rotter et al. (2009). Three replicate plants of 96 individuals (total 288 plants) including 93 backcross progeny individuals along with the colonial x creeping hybrid (TH15) parent, the creeping bentgrass parent (9188), and the creeping bentgrass grandparent (5061) were exposed to drought stress in three different environments (the colonial bentgrass grandparent was not available for testing). The environmental conditions (location and year) used for the drought stress treatment included greenhouse trials in May of 2009 (GH2009) and March of 2010 (GH2010) and a growth chamber trial during February of 2010 (GC2010) in New Brunswick, NJ. Average day night temperatures in the greenhouse were 21/18 in May 2009 and 18/16 in March 2010. Natural sunlight supplemented with sodium lamps allowed for approximately 800-1000 μmol m^{-1} s^{-1} photosynthetically active radiation (PAR). The growth chamber environment conditions were set to maintain 23/20 day/night temperatures, 14 hr light period set to 500 μmol m^{-1} s^{-1} PAR, and 60 % relative humidity.

Watering Treatment

For both GH 2009 and GH2010, tillers of the test plants were allowed to establish and were hand trimmed to a canopy height of approximately 2.54 cm until the canopy covered the entire soil surface of the pots (13.97 x 13.97 cm). All plants were given an equal volume of slow release granular fertilizer prior to watering treatments. The growing media was a Canadian Sphagnum Peat Moss, perlite, vermiculite, and limestone mix (Fafard® 2; Agawam, MA). Prior to watering treatment imposition, equality of the starting soil volumetric water content (SWC %) of each pot was ensured by placing all pots in trays filled with water to allow soaking for 5 mins. After the soil to reached maximum field capacity, the pots were then allowed to drain on a mesh greenhouse bench.SWC was determined by the time domain reflectometry method (Topp et al., 1980) using a Trase TDR instrument (Soil Moisture Equipment Corp, Santa Barbara, CA) attached to 8 cm three-pronged waveguide probes. Initial SWC values were approximately 25% and were not significantly different among pots. Three replications of the population were exposed to drought stress and three were maintained under well-watered conditions. For the drought stress treatment, water was completely withheld for approximately two weeks for both trials until the SWC reached approximately 5%.

For the GC2010 study, three replications of the population were grown in 6 large containers (60 cm x 120 cm x 30 cm deep) filled with a sterilized 1:1 mixture of pure sand and a fine-loamy mixed mesic type Hapludult soil with 10 one cm^2 diameter holes drilled in the bottom for adequate drainage. Two

containers held one replicate of the entire population (approximately 50 plants per container). This experimental design allowed all plants to grow in uniform soil moisture to eliminate possible soil moisture variation in individual pots that would confound evaluation of genetic variation. The plants were established in the growth chamber from small plugs (approximately 20 tillers/plant) and were hand trimmed to a canopy height of approximately 3 cm until they filled in a 7.62 cm^2 area. Plants were maintained separately by trimming the edges of the plants to remove creeping stolons. All containers were well watered during establishment (approximately one month) to maintain SWC at approximately 25%. For water stress treatment, water was completely withheld until the SWC reached 5%. The SWC was tracked throughout the duration of the study by TDR and no significant difference in drying rates occurred between the containers (data not shown).

Analysis of Physiological Traits for Drought Tolerance

Relative water content (RWC), turf quality ratings (TQ), electrolyte leakage (EL), and canopy temperature depression (CTD) of leaves were measured during all trials (GH2009, GH2010, GC2010). Leaf RWC was calculated based on fresh (FW), turgid (TW), and dry weights (DW) of approximately 0.1 g of leaf sample using the following formula: (FW–DW)/(TW–DW) x 100. Leaf FW was determined on a mass balance immediately after being excised from the plants. TW was determined after soaking the leaves in deionized water for 12 h in a closed Petri dish at 4°C and weighed immediately after being blotted dry. Leaves were then dried in an 80°C oven for at least 72 h prior to weighing for DW (Barrs

and Weatherley, 1962). Turf quality (TQ) was rated throughout drought treatment based on canopy uniformity, wilting, color, and density on a scale of 1 to 9, with 1 being brown, desiccated grass and 9 being healthy green, dense grass (Turgeon, 2008). Measurements of leaf EL were performed by taking approximately 5-10 leaves from each plant, rinsing the leaf surface in deionized water, soaking them for 24 h on a shaker, and measuring the conductivity of the immersion water (C_i). Samples were then placed in an autoclave to kill the tissue and placed on a shaker for 24 h. The conductivity of the immersion water (C_{max}) was then determined. EL was calculated as C_i/ C_{max} x 100 (Blum and Ebercon, 1981). Leaf chlorophyll content (CHL) was determined in the GH2010, GC2010 trials by extraction of total chlorophyll and by determining the relative leaf chlorophyll content non-destructively with a chlorophyll meter gun (CM 1000, Spectrum Technologies, Inc.; Plainfield, IL, USA). Total CHL was extracted in the dark for 72 h in dimethyl sulphoxide from approximately 10 leaves per plant. The absorbance of the leaf extract was measured at 663 nm and 645 nm with a spectrophotometer (Spectronic Genesys 2; Spectronic Instruments, Rochester, NY, USA). CHL was calculated using the formula described in Arnon (1949) based on leaf DW. The normalized difference vegetation index (NDVI) was determined during trials GH2010 and GC2010 using a FieldScout TCM 500 NDVI Turf Color Meter (Spectrum technologies, Inc., Plainfield, IL, USA).

Experimental Design and Statistical Analysis

The experimental design was a randomized block design with irrigation treatment as the main plots and plant materials randomized within the watering

treatment block with three replicates for each irrigation treatment and grass

material. For analysis of the physiological phenotypic data, effects of watering

treatment, plant materials, and corresponding interactions were determined by

analysis of variance according to the general linear model procedure of SAS

(Version 9.0; SAS Institute, Cary, NC). Differences between watering treatments

and plant means were separated by Fisher's protected least significance difference

(LSD) test at the 0.05 probability level. When genotype effects were significant,

the traits were subsequently used for QTL analysis. Raw means, adjusted means,

and genotypic variance per trial were calculated for each physiological trait using

the PROC MIXED procedure in SAS (Version 9.0; SAS Institute, Cary, NC).

Broad-sense heritability (H^2) estimates of TQ, EL, CHL, RWC, NDVI, and CTD

were calculated using the random model of the PROC MIXED procedure in SAS

version 9.0 (SAS Institute, Cary, NC) from restricted maximum likelihood

(REML) variance and covariance components (Bonos, 2006).

The colonial bentgrass linkage map that was used to detect QTL was

described in Rotter et al. (2009). The map consisted of 110 gene-based markers

and 212 AFLP markers. For detection of QTLs and analysis of the statistical

significance of QTL regions, the software MapQTL 5.0 (Kyazma ® software ;

van Ooijen, 2004) was used to conduct Kruskal Wallis (KW), interval mapping

(IM), automated co-factor selection (ACS) (a forward-backward method of

stepwise regression), MQM mapping procedures, and permutatuion analysis. QTL

analysis was performed using the mean values for each phenotypic trait for each

environment and year. Interval mapping (Lander and Botstein 1989; Jung et al.,

1996) was used to detect the location of QTLs and determine the magnitude of their effects based on the percentage of phenotypic variance explained (Jansen and Stam, 1994; Zeng, 1994). KW was used to determine whether markers were significant when evaluated individually in a nonparametric based analysis. Following KW and IM, potential markers linked to QTL were selected and ACS analysis and MQM mapping were performed ($P = 0.02$). Permutation analysis was conducted for all traits in each year independently with 1000 iterations to determine LOD significance thresholds for each trait at the genome-wide $P \leq 0.05$ level (Churchill and Doerge, 1994). Markers with LOD values that were below the genome-wide threshold LOD but were consistently significant by KW analysis were deemed putative QTLs. QTLs were classified and discussed as major ($R^2 > 10$) or minor ($R^2 < 10$) as discussed by Collard et al. (2005 and references therein).

RESULTS

Phenotypic Trait Analysis

The mean values of the parents of the backcross population were significantly different for most of the traits measured except for RWC and CTD and all mean values of all traits declined significantly in response to stress for both the parents and the progeny (Table 1). The frequency distributions for two major representative traits, TQ and RWC, further illustrate the response of the parents and population to drought stress treatment (Figure 1). Other traits exhibited the same frequency distribution pattern (data not shown).

The hybrid parent TH15 had greater mean values of TQ, RWC, NDVI and lower values of EL on several sampling dates compared to the creeping bentgrass parent 9188 or creeping bentgrass 5061 grandparent. The creeping bentgrass grandparent was only significantly different from both parents for the traits CHL during MD and CTD during SD. The backcross progeny exhibited continuous trait distributions indicating significant genetic variation to produce transgressive segregation within the population for all three trials (GH09, GH10, GC10). This also illustrates that the phenotypic traits measured (TQ, CTD, CHL, RWC, EL and NDVI) were quantitative in nature. Within the population there was a range of values across all years which were altered by drought stress severity. For example, the range in TQ was 7 to 9 with a mean of 8.86 and for RWC there was a range of 82 to 87% RWC with a mean of 83% during well-watered conditions; these values changed to a range of 1 to 9 with a mean of 2.9 for TQ and a range of

6.2 to 92% with a mean of 38% RWC during SD (Figure 1, Table 1). The broad

sense heritability was the highest for TQ 0.64 and 0.58 under MD and SD,

respectively and ranged from 0.25 to 0.48 for the remaining traits.

Identification of Quantitative Trait Loci (QTL)

A total of 32 markers were significantly associated with putative QTLs on

7 different chromosomes (1A1, 1A2, 2A1, 2A2, 5A1, 5A2, and 6A2) (Table 2,

Figure 2). The traits associated with the identified QTLs are as follows: CHL was

associated with regions on chromosome 1A1, 2A1, and 5A2; TQ with 2A2, 5A1,

5A2, 6A2; CTD with 1A1, 2A1, 2A2; EL with 2A1; NDVI with1A2 and 2A1;

RWC with 5A1 and 5A2. Chromosome 2A1 harbored the greatest number of

markers and the greatest amount of overlap between experiments and traits that

were associated with possible QTL regions. Each QTL was not significant in all

locations, however, several regions were repeatedly detected among trials (Table

2).

The traits CHL, CTD, EL, and NDVI overlapped in the region at

approximate 32-60 cM on chromosome 2A1. On chromosome 2A2, TQ and CTD

were both associated with the location of approximately 53-68 cM. The QTLs

identified on this chromosome 2A2 ranged in LOD score from 2.62 to 4.23.

Chromosome 1A1 had significant QTL for CHL and CTD at the same location,

which included the region from 2-10 cm. Chromosomes 5A1 and 5A2 also had

QTL with significant overlap among traits since TQ and RWC were associated

with the same region on 5A1 (location 3-10 cM) and TQ and RWC (23-30 cM),

TQ and CHL (69-76cM) were found on chromosome 5A2. Chromosome 6A2

had two QTLs (TQ and RWC) that both flanked the same marker

(n313_ACT_TAA). QTLs at the markers FE597043 (chromosome 1A1),

DV855508 and AAG_CAA3 (chromosome 2A1), DV855155 (chromosome 2A2),

DV859329 (chromosome 5A1), ACG_CAG2 (chromosome 5A2), and

n313_ACT_CAA (6A2) were all found in repeated trials. For the majority of

QTLs the additivity value was positive whereas all QTLs on chromosomes 5A1

and 5A2 had negative additive values. Classification of the QTLs by the R^2 value

(phenotypic variation explained %) revealed 31 major and 1 minor QTL. The

minor QTL was on group 2A1 at position 39 and associated with the marker

n282_ACG_CAA.

DISCUSSION

Phenotypic traits of TQ, CHL, NDVI, CTD, and RWC are positively associated with drought tolerance in cool-season grasses and have been used widely for the selection of drought tolerance (Huang, 2004; Bonos and Huang, 2006). Maintenance of TQ, CHL, NDVI, and RWC under drought stress are considered either primary or secondary factors that may result from the ability of plants to maintain cellular hydration and health by multiple defense responses which may lead to less drought damage, maintenance of chlorophyll, the ability for continued growth, or other cellular defense mechanisms (Fry and Huang, 2004). The ability of plants to maintain greater values of CTD under drought stress conditions is associated with enhanced drought tolerance since it may be related to greater transpiration for cooling and continued growth under stress conditions (Blum, 2009). EL is negatively correlated with drought tolerance since greater levels of EL indicate greater cellular membrane damage due to drought stress (Blum and Ebercon, 1981).

Phenotyping the parents and F_2 progeny of the colonial and creeping bentgrass hybrid population revealed that genetic (based on additivity and heritability values) and phenotypic (based on frequency distributions and means) variability exists for the traits TQ, CHL, CTD, EL, NDVI and RWC. The mean values for each trait of the creeping 5061 grandparent, the colonial x creeping hybrid TH15, the creeping bentgrass 9188 parent, and the backcross population exhibited significant differences. Generally, few significant differences occurred

for phenotypic parameters between the creeping grandparent 5061 and the creeping bentgrass parent 9188. However, the TH15 colonial hybrid exhibited more drought tolerance traits than the creeping parent 9188 or the creeping grandparent 5061. Previous work has shown the drought tolerance characteristics of colonial and creeping bentgrass, particularly in TQ and RWC, are similar under drought stress (DaCosta and Huang, 2006). Rotter et al. (2009) noted that interspecific hybridization of creeping and colonial bentgrass may be a viable way to introgress disease resistance genes from colonial bentgrass into creeping bentgrass, while maintaining the quality in turf performance characteristics of creeping bentgrass. Based on the better phenotypic attributes of the TH15 hybrid relative to the creeping parent and creeping grandparent under drought stress, this interspecific hybridization may also be a way to enhance the drought performance of the bentgrasses. It is worth noting that the relative drought tolerance of creeping and colonial bentgrasses are highly cultivar specific and both have been described as having relatively low drought tolerance (Morris, 2008).

Evaluation of additivity of a given trait indicates whether having a genotype of a given parent is more associated with either drought tolerance or sensitivity. In this study, positive additivity values indicate a positive association between having the QTL marker (a genotype from parent TH15 hybrid) and an increase in the trait value. QTLs detected in this study for most traits had a positive additivity value except for the traits TQ and RWC on groups 5A1, 5A2, 6A2 where negative additivity values were detected. Negative additivity indicates that not having the marker (genotype of creeping bentgrass 9188) correlates with

an increase in the value of the trait. Therefore, having the markers (colonial bentgrass genotype) for TQ and RWC on group 5A1 and 5A2 (n390_AGC_CTA and DV859329 on group 5A1 and ACG_CAG2 on group 5A2) may be related to drought sensitivity while all other QTLs may indicate that having the colonial bentgrass genotype in a given region may promote drought tolerance.

Heritability is an estimate of how much of the phenotypic variation seen in a population is due to genetic factors (Visscher et al., 2008). The heritability values of the phenotypic traits were generally calculated to be approximately TQ> CHL, CTD, EL> RWC, NDVI. TQ was the most heritable trait, which is expected since TQ could be considered the most complex physiological trait measured in the study; TQ is based on multiple factors such as leaf density, color, and degree of wilting, and therefore should have the most genetic basis underlying the trait. The lower values of heritability detected such as for RWC and NDVI could be due to the confounding effects of multiple adjacent QTLs, which would contribute to a higher residual variation and error values (Kearsey and Farquhar, 1998). Similarly, relatively low heritability values were detected for RWC in drought stressed rice plants (Courtois et al., 2000) and NDVI in heat and drought stressed barley (Pinto et al., 2010). Enhancing the estimation power of heritability of the QTLs to improve their use in determining other factors such as the response to selection could be achieved by increasing the replications used in future studies and increasing the map resolution for these specific traits (Kearsey and Farquhar, 1998).

Creeping and colonial bentgrasses are allotetraploid species (2n=4x=28), however, the creeping bentgrass chromosome set was determined to be the $A_2A_2A_3A_3$ type and colonial bentgrass is of the $A_1A_1A_2A_2$ type. Rotter et al. (2010) recently proposed that velvet bentgrass (*Agrostis canina* L.) was the A_2 genome donor. The diploid origins of the A_1 and A_3 genomes are not known. A total of 32 QTLs were detected on 7 groups. Since potential QTLs with both positive and negative additivity values were detected on both A1 and A2 types, it does not seem that drought tolerance or sensitivity may be related to chromosomal origin directly. Specific QTLs were found on groups 1A1, 1A2, 2A1, 2A2, 5A1, 5A2, 6A2 and the closest associated markers with these QTL regions had an average LOD score of 3.29. The range of percentages of the variation in phenotype explained by the marker (phenotypic variance explained %) of each QTL was detected. Therefore the QTLs were major or minor in nature. Major QTL that were repeatedly detected in each trial may have the greatest impact on imparting drought tolerance and may be the most useful in future breeding strategies.

Comparison of QTLs detected here to homologous chromosomes and closely related species such as rice reveals both distinguishing factors and regions that may be syntenous. Rice chromosome 1 has been found to contain genes for drought tolerance associated with leaf rolling and leaf drying (Salunkhe et al., 2011). No significant QTLs were detected on groups 3A1 or 3A2, which is syntenic to rice chromosome 1 (La Rota and Sorrells, 2004). Lilley et al. (1996) found QTLs associated with osmotic adjustment during cellular water deficit on

rice chromosomes 1, 3, 7 and 8. Group 2A1 from the colonial bentgrass linkage map used in this study contains the most similar genes to rice group 7. Many markers from group 2A1 were associated with drought stress in this study and could be related to those of Lilley et al. (1996). Kato et al. (2007) found QTLs related to relative growth rate and water use efficiency during drought stress of rice seedlings. The most significant QTLs detected here were on colonial bentgrass linkage groups syntenic with rice chromosomes 2, 4, 5, and 7 (Figure 2, 3) (Rotter et al., 2009; La Rota and Sorrells, 2004). Since the most markers with the greatest significance in this study occurred in group 2A1, which is syntenic to rice chromosomes 4 and 7, the results could be consistent across these studies. Meta-analysis may be useful in the future to evaluate these similarities in findings as well as a comparison across QTL studies relating to identifying QTL for drought tolerance traits.

Specific genes associated with the markers listed in Table 2 may not be directly responsible for the QTL or phenotypic variation observed due to the trait, since QTLs are large genomic regions. However, these genes could potentially be related to the trait governed by the QTL and play a role in the phenotypic variation observed or the enhanced drought tolerance. Therefore, the functions of these specific genes are discussed as they relate to the specific trait and drought tolerance for those markers that were repeatedly detected in multiple trials.

Marker DV857259 within the possible QTL for NDVI located on chromosome 1A2 was significant under well watered and drought stress

conditions and could be associated with a gene coding for a ketol acid reductoisomerase. Various types of ketol acid reductoisomerases are primarily involved in carbohydrate metabolism and have been associated with the drought response. For instance, transcripts of chloroplast ketol acid reductoisomerase exhibited an increase fold-change in plant types of rice exhibiting relatively good drought tolerance and a high degree of osmotic adjustment during drought stress (Hazen, et al., 2005) and were differentially expressed due to drought stress in proteomics studies of rice (Shu et al., 2011). Under well watered conditions, the involvement of ketol acid reductoisomerases in carbohydrate metabolism could be related to the variation in genetic color attributes or growth rate among the colonial and creeping bentgrass hybrids. This QTL may have value as a potential marker for the detection of aesthetic qualities and fast growth characteristics in turfgrass germplasm to promote selection of grasses with adequate canopy establishment characteristics under optimal conditions. This region may warrant further investigation of additional phenotypic traits that may also associate with this area of the genome.

During drought stress, stomatal closure may decrease intercellular CO_2 to limit photosynthethic reactions. Concomitantly, a down-regulation of photosynthesis and Calvin cycle processes causes an increase in photo-damage due to energy absorption exceeding the rate of energy use and reactive oxygen species generation. Thus, the efficiency with which electron transport machinery in both chloroplasts and mitochondria can process excess energy is reduced by both stomatal and non-stomatal factors during drought stress (Aro et al., 1993;

Chaves et al., 2009). Maintenance of chloroplastic and mitochondrial proteins involved in energy metabolism, photorespiration, and degradative enzymes serving to remove, replace, or repair damaged photosystem II complexes and other components of the electron transport chains have been associated with enhanced drought tolerance of various plant species (Merewitz et al., 2011; Rivero et al., 2010; Chaves et al., 2009 and reference therein) and have been associated with cultivar variation in drought tolerance (Ford et al., 2011). The identities of three gene-based markers linked to QTL regions on chromosomes 2A1 and 2A2 for CTD (DV855554 and DV853200) and CHL (DV859491) may indicate these regions are associated with energy metabolism.

Another QTL region for TQ and CTD numbered DV855155 on group 2A2 was repeatedly detected and may also be associated with energy metabolism. A potentially linked gene codes for an oxygen evolving enhancer protein (OEE). OEEs are primarily located in chloroplast electron-transport chain proteins complexes (Kusnetov et al., 1994) and OEEs may relate to drought stress tolerance by promoting PS II stability under stress and maintaining the flux through the electron transport chain (Koichi et al., 2000). Maintenance of PS II reaction centers in chloroplasts may be related to the same tolerance mechanism, promoting photosynthesis and photochemical quenching during drought stress (Giardi et al., 1996). PSII proteins exhibit considerable changes in activity, efficiency, and can undergo structural rearrangement or be degraded during drought stress. PSII activity and efficiency is known to be related to the amount of available CO_2 assimilated, energy dissipation, and photorespiration rates

(Yardanov et al., 2003). How PSII proteins may relate to CTD are unclear. However, it may suggest a link between higher CTD and stomatal opening with more available CO_2 to affect the required content of PSII subunits, the degree of degradation of PSII reaction centers, and the necessity for processes in photorespiration. The results suggest that important chromosomal regions in creeping and colonial bentgrass may be those related to energy generation and utilization under drought stress.

The QTL associated with the marker DV855508 on chromosome 2A2 is perhaps the most reliable QTL detected since it was located using multiple traits (EL, CTD, CHL) and in multiple trials. The same overlapping region on chromosome 2A2 was significant for the AFLP markers n282_AGC_CAT and n73_ACG_CAA for CHL. The marker sequence DV855508 shows significant alignment with a small nuclear ribonucleoprotein (snRNP) in the database. SnRNP proteins regulate nuclear receptor activity thereby effecting mRNA metabolism. In regards to drought stress, snRNPs have been implicated in conferring drought tolerance by enhancing the sensitivity of plants to ABA and thereby have a role in controlling stomatal closure during drought stress (Xiong et al., 2001). Thus, the presence of the snRNP marker may confer drought tolerance to grasses via regulation of ABA and stomatal aperture. Further investigation of the specific type of snRNP and its downstream target of this gene may be warranted.

Information regarding molecular markers and QTLs for drought tolerance traits is desirable in the breeding of grasses and the genomic information can also be used in other closely related crop species. Chromosomal regions identified here may be useful in future studies to further characterize important genomic regions associated with drought tolerance. Fine mapping and other molecular techniques to elucidate specific genes within these important chromosome regions may be beneficial in deciphering genetic control of important drought tolerance characteristics and how they may relate to phenotypic variation of drought tolerance in various plant species. Further dissection of these QTL regions may be used in the future for marker assisted selection in breeding programs and for further investigation of genetic control of drought tolerance in grasses and related species.

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TABLES AND FIGURES

Table 1 Means of parental lines and the F_2 progeny with the calculated genotypic variance and heritability (H^2) estimates for each physiological trait (TQ, turf quality; CHL, chlorophyll content; EL, electrolyte leakage; CTD, canopy temperature depression; RWC, relative water content; NDVI, normalized difference vegetation index) at each level of water stress (W = watered, MD= moderate drought, SD = severe drought) where significant genotypic interactions occurred. P1=parent 1; P2= parent 2; GP= grandparent. † Values followed by the same letter within a column are not significantly different ($P \geq 0.05$) based on a LSD test.

	TQ		CHL		EL		CTD		RWC		NDVI		
	MD	SD	MD	SD	MD	SD	MD	SD	MD	SD	W	MD	SD
Mean creeping 5061 GP	6.11 c†	4.00 a	270.3 a	225.7 a	48.6 b	92.7 a	2.94 b	4.75 a	69.33 b	22.74 c	0.76 a	0.76 a	0.72 a
Mean P1 creeping 9188	6.22 c	2.87 b	243.7 b	210.3 a	36.7 c	94.2 a	2.32 b	3.85 b	68.83 b	40.52 b	0.70 b	0.73 a	0.70 b
Mean P2 Hybrid TH15	7.00 b	3.4 ab	238.0 a	210.7 a	25.9 c	62.5 b	1.02 b	3.58 c	72.01 a	45.67 a	0.75 a	0.74 a	0.75 a
Mean F2	8.72 a	2.90 b	241.8 b	208.8 a	68.3 a	58.4 b	8.72 a	3.76 b	58.77 c	37.98 b	0.64 c	0.69 b	0.63 b
Min/max Range F2	1/9	1/9	126/343	119/266	18.7/78	20/100	-4.6/5.1	-3.5/10.7	6.0/80.0	6.2/92.1	0.6/0.8	0.52/0.8	0.36/0.8
Gen var F2	1.85	1.57	1213.0	955.0	60.50	41.6	1.18	0.78	44.86	39.70	0.002	0.004	0.004
Broad sense H²	0.64	0.58	0.46	0.43	0.48	0.32	0.44	0.31	0.25	0.29	0.04	0.29	0.25

Table 2 Chromosomal locations of markers and QTL regions for each trait associated with drought tolerance or sensitivity. Marker names given are those that are the nearest and most significant marker to a given QTL. Gene information is listed for the closest associated marker, however, this does not indicate that the genes are responsible for the QTL. Unknown gene information indicates no good match in the Genbank database. †detected under well-watered conditions

Chrom #	Pos	Marker name	Trait	Trial	LOD	K*	Phenotypic variance (%, R²)	Additivity	Gene information of closest associated marker
1A1	9	FE597043	CHL	GH10	3.01	11.025	13.8	1.1	DVL peptide
			CTD	GC10	3.15	10.65	18.7	0.28	DVL peptide
1A2	6	DV857259	NDVI†	GH10	3.04	11.29	14.3	0.03	Ketol-acid reductoisomerase (type 1)
			NDVI	GH10	3.12	12.54	12.3	0.05	Ketol-acid reductoisomerase (type 1)
2A1	10	DV855554	CTD	GH09	2.92	12.129	13.4	0.72	10kD PSII protein
	12	DV853200	CTD	GH09	2.94	13.18	13.6	0.73	Rieske iron-sulfur protein, chloroplast
	22	DV853949	CHL	GH10	3.58	12.89	16.3	14.10	Glutamine synthetase
	36	n73_ACG_CAA	CHL	GH10	3.18	10.79	14.6	12.81	-
	38	DV855508	EL	GH09	3.25	13.36	14.9	8.80	snRNP protein
			CTD	GH09	4.19	17.58	18.8	1.69	snRNP protein
			CHL	GH10	3.3	14.90	13	4.42	snRNP protein
	39	n282_AGC_CAT	CHL	GH10	3.33	15.20	9.395	25.79	-
	43	AAG_CAA3	CHL	GH10	3.43	13.48	18.9	13.44	-
			NDVI	GH10	2.62	12.89	12.1	0.013	-
	45	n302_ACT_CAC	CHL	GH10	4.23	8.639	18.9	13.61	-
	50	DV859491	CHL	GH10	3.01	6.687	13.8	12.44	ATPase family (AAA)
	52	n226_ACC_CTA	CTD	GC10	2.93	10.12	13.5	0.19	-
	56	n191_ACT_CAG	CHL	GC10	3.13	14.3	13.2	0.015	-
	59	ACG_CAG6	NDVI	GH10	3.18	10.42	14.6	0.029	-
	80	n65_ACC_CTC	NDVI	GH10	3.19	12.54	14.6	0.015	
	62	n163_ACC_CTC	NDVI	GH10	4.12	10.70	22.4	0.018	-
2A2	50	DV856409	TQ	GH09	3.19	13.05	14.6	0.44	Ubiquinol-cytochrome c reductase iron-sulfur subunit
	59	DV855155	TQ	GH10	3.47	11.23	19.1	4.99	Oxygen-evolving enhancer protein 1
			CTD	GH09	3.08	15.78	15.9	0.76	Oxygen-evolving enhancer protein 1
5A1	1	n390_AGC_CTA	RWC	GH10	3.27	9.29	15.7	-6.18	-
	8	DV859329	TQ	GH10	3.63	11.54	16.5	-1.21	One helix protein
			RWC	GH10	3.23	17.5	14.8	-5.99	One helix protein
5A2	25	ACG_CAG2	RWC	GH10	3.13	9.838	14.3	-1.05	-
			TQ	GH10	4.38	16.93	19.5	-1.47	-
	70	n120_ACC_CTA	TQ	GH09	3	11.2	16.2	0.26	-
	76	DV857545	CHL	GC10	3.19	12.34	19.5	0.29	Unknown protein
6A2	33	n313_ACT_CAA	TQ	GH09	3.34	15.2	13.91	11.21	-
			RWC	GC10	3.01	8.404	10.91	9.87	-

Figure 1 Population wide frequency distributions for the major traits A) turf quality (TQ, 1-9 scale rating) and B) relative water content (RWC) on a given year (GH2009, GH2010, GC2010) at a given level of drought stress (MD=moderate drought, SD=severe drought). The mean values for each parent are indicated by arrows with respective error bars only present when a significant difference was detected. Open arrows indicate the mean of the creeping bentgrass parent 9188 and closed arrows indicate the mean of the hybrid bentgrass TH15 parent.
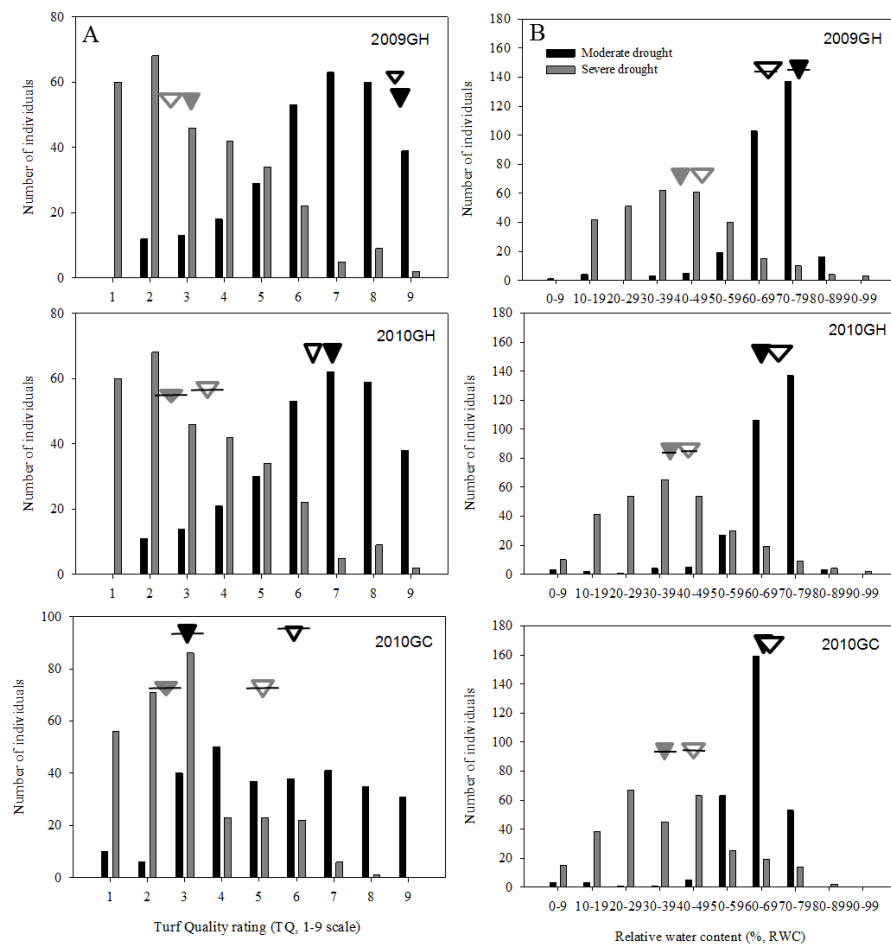
Figure 2 Chromosomal locations of quantitative trait loci (QTL) on groups 1A1, 1A2, 2A1, and 2A2 identified for the traits indicating drought tolerance or sensitivity on the genetic linkage map showing the amplified fragment length polymorphisms (AFLP; identified by marker number and selective nucleotides) and expressed sequence tag (EST; identified by Genbank accession numbers) markers of colonial bentgrass (adapted from Rotter et al., 2009). The numbers in parenthesis indicate the rice chromosome number exhibiting synteny to the marker and asterisks indicate distorted segregation (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$).
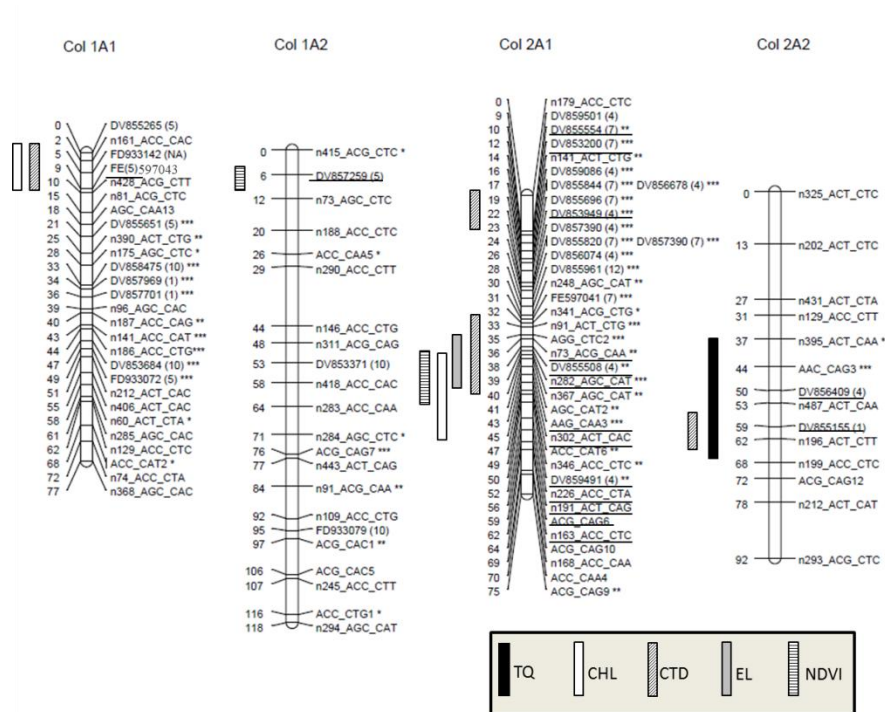
Figure 3 Chromosomal locations of quantitative trait loci (QTL) on groups 5A1, 5A2, and 6A2 identified for the traits indicating drought tolerance or sensitivity on the genetic linkage map showing the amplified fragment length polymorphisms (AFLP; identified by marker number and selective nucleotides) and expressed sequence tag (EST; identified by Genbank accession numbers) markers of colonial bentgrass (adapted from Rotter et al., 2009). Asterisks indicate distorted segregation (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$).
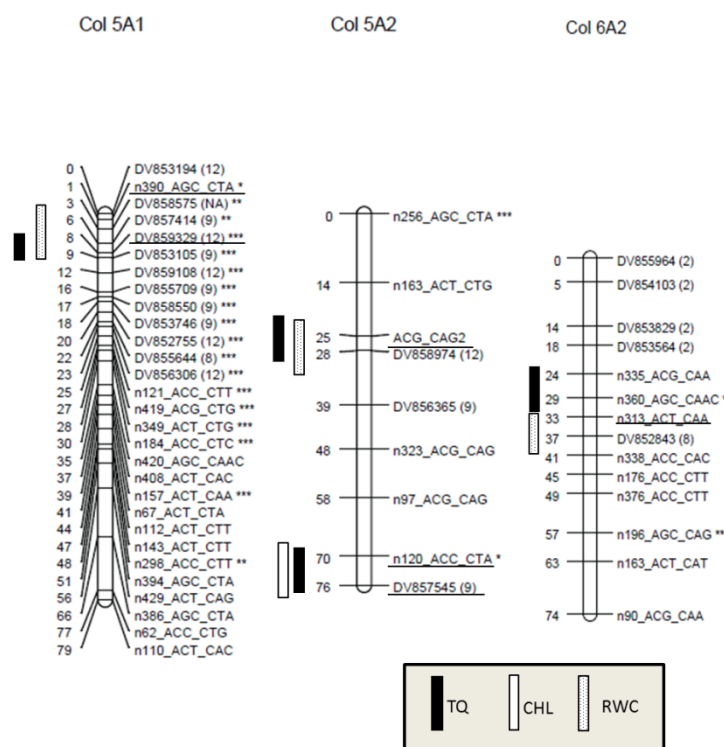
CONCLUSIONS

The goals of the dissertation were to provide new insights into various aspects of the drought response of turfgrasses through evaluation of genetic, proteomic, metabolomic, and genomic attributes. The plant germplasm that was evaluated included non-trangenic and transgenic creeping bentgrass plants as well as a hybrid creeping x colonial bentgrass population. The intrinsic variation in drought tolerance that existed among these plants allowed for understanding which phenotypic traits are associated with improved drought tolerance in turfgrass species and the potential underlying biochemical mechanisms responsible for those traits.

The objectives of Part I was to evaluate the responses of creeping bentgrass plants containing a transgene that promotes cytokinin biosynthesis under drought stress. The results of this work suggest that enhanced cytokinin content improves creeping bentgrass growth under drought stress conditions. Major responses to this altered hormone balance include a reduction of leaf senescence (e.g. chlorophyll content maintenance), delayed cellular water loss (improved relative water content), improved energy metabolism (e.g. photosynethesis and respiration rates), maintenance of membrane integrity (e.g. less lipid peroxidation), promotion of stress defense enymes (e.g. antioxidants), accumulation of compatible solutes (e.g. free amino acids, sugars and other small organic compounds). Cytokinin-induced maintenance of the health of antioxidants, photosystems, and a rapid induction of glycolysis could have

manifested into the aforementioned traits such as osmotic adjustment, lipid health, and maintenance of carbon metabolism. Major findings related to specific cytokinin-induced responses are discussed below.

Specific pathways discussed in the dissertation that consistently showed differentially expression in the transgenic plants compared to non-transgenic plants at the gene, protein, and metabolite level were identified. For instance, within carbon metabolic pathways, transgenic plants exhibited maintenance of gene transcripts and RubisCo protein subunits, RuBisCo activase, and photosystem II transcript and protein subunits. In respiratory pathways, GAPDH transcripts and proteins were consistently maintainted to a greater extent in transgenic plants. These changes could be associated with the observed promotion of photochemical efficiency and with maintenance of various intermediate metabolites in the photosynthetic and respiratory pathways such as sugars (glucose, sucrose, fructose) and organic acids (citric acid, malic acid) in the citric acid cycle. Secondary pathways and metabolites that are less well known in how they may relate to cytokinin regulation and drought stress were also identified. For instance, maintenance of transcripts and proteins related to membrane transport (ABC transporter, aquaporin, ATPases) and unknown proteins were found in transgenic plants. Metabolites totaling 45 included those that are well known in the drought response and those that are less well studied. For instance, carbohydrates (sucrose, fructose, maltose, and ribose), amino acids and amine compounds (γ-aminobutyric acid, alanine, and glycine), and organic alcohols (glycerol, myo-inositol) were all maintained to a greater extent in transgenic

plants. Taken together, the results of genetic, proteomic, and metabolic analysis suggest several molecular pathways that may be most responsible for the increased drought tolerance phenotype in the transgenic plant. These may be viable pathways to target in future work to further enhance the understanding of drought tolerance and for utilization for improving plant drought tolerance.

The other approach taken with the aim for the results to be used in future plant breeding strategies to improve plant drought tolerance was discussed in Part II of the dissertation. The research goal of Part II of the dissertation was to identify genomic regions controlling important phenotypic traits that may be associated with improved drought tolerance of turfgrasses also. This was achieved by evaluating a colonial bentgrass (*Agrostis capillaris* L.) x creeping bentgrass (*Agrostis stolonifera* L.) population that segregated for drought tolerance characteristics. A total of 32 potential QTLs of varying effects were detected on 7 chromosomes. Chromosomal regions containing significant overlap of QTLs was found for traits indicative of drought tolerance such as maintenance of chlorophyll content, canopy temperature depression indicating transpiration, cellular membrane stability, and green leaf biomass of the turf canopy. Similar to part I, these regions may play a role in governeing drought tolerance in *Agrostis* species. Potential genes in the QTLs regions may for these physiological traits indicate that the QTLs may be linked to metabolic factors involved in nitrogen metabolism and energy metabolism such as photosynthesis and respiration. The QTL regions identified here could contain important genetic factors conferring drought tolerance in bentgrass species.

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Publications

JOURNAL ARTICLES

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BOOK CHAPTER

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