MECHANISMS OF POLYAMINE-INDUCED ABIOTIC STRESS TOLERANCE IN CREEPING BENTGRASS (*AGROSTIS STOLONIFERA*)

By

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The turfgrass industry, which includes home lawns, golf courses, and parks, is a 40 billion-dollar industry in the United States. Many turfgrasses require high inputs due to functionality requirements and susceptibility to abiotic and biotic stresses. For instance, many turf areas are in salt prone environments, such as shorelines, salt affiliated soils, or areas required using reclaimed water. In addition to salt stress, drought stress, due to limited or restricted irrigation, frequently occurs in turfgrasses. Creeping bentgrass is a widely used cool-season turfgrass species, particularly on golf course putting greens. In this thesis, we aimed to evaluate whether a metabolite pathway, the polyamine biosynthetic pathway, could be exploited to develop an environmental friendly and cost effective chemical product to increase abiotic stress tolerance. We also intended to better understand how these metabolites may play a role in creeping bentgrass stress tolerance.

Polyamines (PAs) including putrescine (Put), spermidine (Spd), and spermine (Spm) are involved in plant growth and stress tolerance. Put and Spd content increased under salt stress in creeping bentgrass leaf tissues indicating that PAs may play a role in creeping bentgrass responses to salt stress. Exogenously applied Spd and Spm improved drought tolerance in creeping bentgrass by maintaining higher leaf relative water content and photochemical efficiency, lower lipid peroxidation, and electrolyte leakage. Gene expression analysis revealed that Spd regulated genes associated with important plant physiological processes such as photosynthesis. Important stress defense pathway genes were also regulated

by Spd application to plants. For instance, genes involved in antioxidant pathways, carbohydrate metabolism, energy metabolism, and cellular response to water deprivation.

ABSTRACT

MECHANISMS OF POLYAMINE-INDUCED ABIOTIC STRESS TOLERANCE IN CREEPING BENTGRASS (*AGROSTIS STOLONIFERA*)

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Creeping bentgrass (*Agrostis stolonifera*) is the most widely used putting green species on golf courses where on shorelines or areas with salt afflicted soils. Recycled and reclaimed water, which can have high salt content, is increasingly being implemented or required for turfgrass irrigation. These practices cause both salt and drought stresses that limit turfgrass health, functionality, and can increase inputs and costs of management. Polyamine (PA) including putrescine (Put), spermidine (Spd), and spermine (Spm) are involved in abiotic stress tolerance in plants. However, their roles in stress tolerance can be highly species specific. It is not well-known whether PA plays a major role in stress tolerance of turfgrass species or whether they can be harnessed and used in turf management industry. Therefore, our specific objectives were to determine PA induced drought and salt stress tolerance mechanisms in plants, to evaluate the effects of salt stress on endogenous PA content in salt stressed and non-stressed plants, and to identify differentially expressed genes by transcriptome analysis due to PA and drought stress treatment of creeping bentgrass.

Under salt stress, leaf Na^+ content increased while leaf K^+ and Ca^{2+} content decreased when compared with non-stressed plants. Salt stress decreased turf quality and leaf osmotic potential but increased leaf electrolyte leakage and canopy temperature depression. Endogenous PA content was altered due to salt stress in creeping bentgrass leaf tissues. Put and Spm content was two to five times higher in salt stressed leaf tissue compared with controls in early salt stressed stages.

Application of a relatively low concentration of Spd (500 $\mu M \cdot L^{-1}$) promoted tillering rates under optimal growth condition in hydroponics. Association of Spd (500 or 750 $\mu M \cdot L^{-1}$) or Spm (500 $\mu M \cdot L^{-1}$) treatment to increased membrane health was revealed as greater photochemical efficiency, higher quantum yield, increased leaf relative water content, less electrolyte leakage, and less lipid peroxidation (malondialdehyde content) in PA treated plants compared to control plants.

Transcriptome analysis using RNA-sequencing evaluated differentially (DE) expressed genes due to drought and Spd application. De novo assembly and transcriptome alignment showed 22% and 19% of genes were either up- or down-regulated due to drought while 20% and 34% of genes were either up- or down- regulated in response to Spd application, respectively. Gene ontology and enrichment analysis indicated that these DE genes were primarily associated with energy metabolism, transport, antioxidants, photosynthesis, signaling, and stress defense processes. This research is the first to provide transcriptome data for creeping bentgrass under drought stress. DE genes identified here could be further investigated for use as molecular markers or for functional analysis in responses to drought and Spd. Since endogenous PAs are regulated by abiotic stress, exogenous application improved abiotic stress tolerance, and genes involved in stress tolerance were differentially expressed due to PA treatment, PA metabolic pathways may be useful in chemical technologies or in breeding strategies that aim to improve creeping bentgrass stress tolerance.

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KEY TO ABBREVIATIONS

ADC: arginine decarboxylase;

DAO: diamine oxidase;

GABA: γ-aminobutyric acid;

ODC: ornithine decarboxylase;

PA: polyamines;

PAO: polyamine oxidase;

SAM: S-adenosylmethionine;

SAMDC: S-adenosylmethionine decarboxylase;

SPDS: spermidine synthase;

SPMS: spermine synthase;

ROS: reactive oxygen species;

ABA: abscisic acid;

GA: gibberellic acid;

JA: jasmonic acid;

DE: differentially expressed;

GO: gene ontology;

Spd: spermidine;

Spm: spermine;

Put: putrescine;

CHAPTER 1

ROLE OF POLYAMINES IN ABIOTIC STRESS RESPONSES

Abstract

Many secondary metabolites in plants are involved in a range of plant physiological processes including abiotic stress tolerance. Major polyamine (PA) compounds including putrescine (Put), spermidine (Spd) and spermine (Spm) have structural, functional, and regulatory activities that allow them to play a major role in abiotic stress tolerance. A thorough understanding of PA function in plant stress tolerance is important for improving plant stress responses, breeding, biomarker development, and other purposes. In this chapter, we discuss the role of PA in several types of abiotic stress and the mechanisms associated with dynamic changes in PA content. Major methods to evaluate PA function are discussed including biosynthetic gene expression, exogenous application of PA, use of PA biosynthesis inhibitors, transgenic manipulation, and development of gain or loss of function mutants.

Keywords: abiotic stress, polyamines, polyamine metabolism, exogenous application, polyamine gene expression, plant stress tolerance

Introduction

Many abiotic stress including water deficit, ion toxicity, temperature differences, wounding, and several others adversely affect plant growth and development. However, plants have evolved several resistance mechanisms to cope with such abiotic stresses, which include escape, avoidance, and tolerance (Mickelbart et al., 2015). Stress tolerance mechanisms are necessary when plants do not have effective escape or avoidance strategies or if the cultural practices of such plants are not allowed for escape or avoidance mechanisms to occur. A major tolerance mechanism to various abiotic stresses is the regulation of gene expression and biochemical pathways controlling polyamine (PA) metabolism. PA is straight chained C3 to C15 aliphatic, hydrocarbons substituted with terminal amino groups (Edreva, 1996). Common PA is essential in higher plants, which include diamine putrescine (Put), triamine spermidine (Spd), and tetraamine spermine (Spm) (Ziegler and Facchini, 2008). Common PAs can exist in different forms in plant cells, for instance thermospermine is a structural isomer of Spm (Kakehi et al., 2008). Some uncommon PAs have been found in different species such as cadaverine and homospermidine in *Leguminosae* (Flores, 1990), canavalmine and pentaamines in sword bean (*Canavalia gladiate*) (Matsuzaki, 1990), norspermidine (caldine) and norspermine (thermine) in alfalfa (*Medicago sativa*) and cotton (*Gossypium hirsutum*) (Kuehn et al., 1990). Homospermine is also described as an uncommon PA found in bacteria and animals, but is not readily found in plants (Smith, 1991). Despite the various types of PAs that have been identified, Put, Spd, and Spm remain the most commonly researched PAs related to stress tolerance.

Whether PAs can be fully classified as plant hormones is debated, but their hormone like function in plants cannot be denied (Davies, 1995). Thus, like any plant hormone, PA

content is precisely regulated in plants. This regulation is achieved by de novo synthesis, inter-conversion, terminal degradation, and transport. PA biosynthesis is part of the tropane alkaloid biosynthesis pathway (Ziegler and Facchini, 2008). They are primarily derived from ornithine or arginine, which are converted by ornithine or arginine decarboxylase (ODC, ADC), respectively, to yield Put. Spd and Spm are formed by the sequential addition of aminopropyl groups to Put and Spd, which is derived from decarboxylated S-adenosylmethionine (SAM) catalyzed by SAM decarboxylase (Kusano et al., 2007; Pang et al., 2007). Based on this PA biosynthesis pathway, many PA biosynthesis genes have been identified, which have allowed for significant insight into the function of PA in plants and how PAs are involved in stress tolerance. *ADC1* and *ADC2* were identified as two arginine decarboxylase encoding genes in Arabidopsis (*Arabidopsis thaliana*) (Galloway et al., 1998). Spm synthase encoding genes *ACL5* and *SPMS* (Hanzawa et al., 2002) as well as Spd synthase encoding gene *SPDS1*-2 were identified (Hanzawa et al., 2000). Additionally, other *SAMDC1-4* were also identified as encoding for S-adenosylmethionine decarboxylase in plants (Fuell et al., 2010; Urano et al., 2004). However, ornithine decarboxylase (*ODC*) is not present in Arabidopsis (Fuell et al., 2010). Further details regarding PA biosynthesis can be obtained from several other reviews focused on this topic (Moschou et al., 2008; Rea et al., 2004).

The basic properties of PAs in plant cells play a major role in PA function in response to abiotic stresses. PAs are positively charged in cells (Krasensky et al., 2012) and are primarily synthesized in plastids and cytosol (Mulangi et al., 2012), meaning they can readily bind to negatively charged ions and membrane proteins (Edreva, 1996). PA also can be conjugated to alkaloids, fatty acids, and hydroxycinnamic acids and such conjugation

contributes to abiotic stress due to its increased bioavailability (Smith, 1991; Pérez-Amador et al., 1996). For instance, increased non-covalently conjugated Spm and Spd, together with covalently conjugated Put and Spd were associated with the tolerance of wheat seedlings to osmotic stress (Liu et al., 2006). Such regulation of many PA biosynthesis genes along with shifts in PA content during abiotic stresses has implicated the significance of PA play in stress tolerance. PA function and regulation has been explored in several model plants and crop species with studies aiming to elucidate complex interactions with major cellular components and hormones that are involved in stress tolerance signaling. Therefore, the goal of this chapter is to provide an update on recent evidence of how PA is associated with abiotic stress tolerance.

Salt stress

Above-optimal salinity in water or soil inhibits plant growth due to imbalanced ion homeostasis in plants. In response to salinity stress, plants can resist salinity damage by accumulation of compatible osmolytes and activation of other stress protective pathways. Regulation of PA content inside of cells has been deemed a salt stress tolerance mechanism used by plants. Several studies aiming to quantify PA content in salt tolerant and sensitive cultivars have been conducted. For instance, endogenous PA concentration in broad bean (*Vicia faba* L.) increased significantly compared with the control after salt stress which indicated the protective role of PAs (Sadak and Abdelhamid, 2015). Similarly, Spd and Spm levels increased while Put level decreased during salt stress and this trend was correlated with salinity tolerance in seven different plant species (Zapata et al., 2004). In Arabidopsis flowers, an increase in salt treatment correlated with an accumulation of free Spd (Tassoni et al., 2008). The same Spd response to salt treatment occurred in maize (*Zea mays*) leaf blade elongation

zone (Rodriguez et al., 2009). Furthermore, in a hydroponics system, Put and Spm increased more in salinity tolerant rice (*Oryza sativa*) cultivar compared to the sensitive one and such increase correspond to induction of PA biosynthetic gene expression (*ADC2*, *SPMS2*, and *SPMS3*) (Do et al., 2014). Another comparative study conducted in foxtail millet (*Setaria italica*) showed elevated free PA content in salt tolerant cultivar and such PA content increase was corresponded to their increased enzyme activities of SPMS and SAMDC under 100, 150, and 200 mM of NaCl stress (Sudhakar et al., 2015). Dynamic changes in PA was also observed in grape plant (*Vitis vinifera*) which showed initial Put increased within a day while increased Spd and Spm levels happened three days after salinity stress (Liu et al., 2011). A similar study was conducted and demonstrated that increased content of Put may have enhanced the synthesis of Spd and Spm in PA biosynthesis pathway under salinity stress (Do et al., 2013). Introducing tritordeum (*Triticum* spp) *SAMDC* cDNA into rice resulted in higher levels of Spd and Spm than wild type plants under NaCl stress and such PA increase was associated with salt tolerance (Roy et al., 2002). Using an opposite approach, anti-sense mutational analysis resulted in degrading the *SPDS* gene in European pears (*Pyrus communis*) showed an increased sensitivity to salinity by the reduction of Spd concentration (Wen et al., 2011) which confirmed the crucial role of Spd in salinity stress alleviation. Taking all of these studies together, there is a clear correlation between Spd and Spm accumulation and salt tolerance.

With regard to the induction of Put in response to salinity stress, Put biosynthetic gene expression level was quantified in response to salinity stress in different degree of salinity tolerant cultivars. Significantly increased *AtADC2* mRNAs were expressed in Arabidopsis (Urano et al., 2003) and increased expression of *MdADC* in apple (*Malus sylvestris*) vitro

callus were observed under salinity stress (Liu et al., 2006). Additionally, salt stress induced the elevation of conjugated Put in a salt resistant rice cultivar while not in the sensitive one. Such effect corresponded to the increased expression activity of *ODC* and *ADC* (Quinet et al., 2010). Salinity stress tolerance and trends in Put accumulation indicate the individual contribution of a PA to stress tolerance. Similarly, a Put biosynthesis insertion mutant of *AtADC2* gene (*adc2-1*) showed reduced free Put content and this *adc2-1* mutant line was more sensitive to salt stress than the wild type plants in Arabidopsis. Such salinity sensitivity in *adc2-1* mutant was recovered by the exogenously applied Put (Urano et al., 2004) which indicated the salinity tolerance effect from Put. Transgenic eggplant (*Solanum melongena*) with an oat *ADC* gene exhibited substantially increased Put content and tolerance to salinity compared to wild type plants (Prabhavathi et al., 2007) which suggested the protective role of Put in response to salt stress.

Whether plants accumulate Put for stress protection or Spd and Spm could be related to species differences and the most prevalent stress tolerance mechanism that plants exhibit. However, modulating the homeostasis of PA through biosynthesis and catabolism in certain species contributes to salinity stress tolerance. Expression of PA biosynthesis (*ADC*, *SAMDC*, *SPDS*, and *SPMS*) and catabolism (*DAO* and *PAO*) genes were up-regulated by exogenous application of PA in orange (*Citrus aurantium*) (Tanou et al., 2014) which showed the association and precise regulation of PA content to trigger PA transduction and oxidative signaling for salt stress defense. Catabolism of Spm may be associated with stress tolerance via γ-aminobutyric acid (GABA) pathways. GABA is a signaling molecule involved in abiotic stress tolerance (Bouché et al., 2004; Beuve et al., 2004; Frak et al., 2002). Besides, Xing et al. (2007) proposed that the catabolism effect of Spm on salinity stress tolerance was due to

promotion of the activity of DAO to stimulate PA degradation. Accumulation of GABA was also found to be induced by salt stress through the Put degradation pathway in brown alga (*Ectocarpus siliculosus*) (Dittami et al., 2011). Therefore, these studies indicate that the balance between PA biosynthesis and catabolism contributes to salt stress tolerance.

The homeostasis of PA during salt tolerance is associated with the reduction of cellular Na^+ due to the differential accumulation of Ca^{2+} and K^+. A plant's ability to maintain K^+ and Ca^{2+} levels while under pressure from Na^+ exposure has been associated with salt tolerance. K^+ is important for maintaining enzyme activities and osmotic adjustment and increased concentration of Ca^{2+} has been shown to improve K^+ nutrition under salt stress (Bacha et al., 2015; Flowers et al., 2015). A double knock-out mutant of Arabidopsis in the *acl5* and *spms* genes could not produce Spm showed higher sensitivity to salt stress than wild type plants. This salinity sensitivity was alleviated by exogenous Spm but not by Put and Spd which suggested the effect of Spm in NaCl stress tolerance. This study also demonstrated that an absence of Spm affected Ca^{2+} levels in the plants (Yamaguchi et al., 2006). Likewise, over expressing *SlSAMS1* in tomato (*Solanum lycopersicum*) showed increased tolerance to alkali stress and such alkali stress tolerance were revealed by increased PA production to trigger essential elements of Ca^{2+}, Mg^{2+}, and Fe^{3+} up take and Na^+ absorption reduction (Gong et al., 2014). Similarly, Spd and Spm accumulation maintains plasma membrane integrity and modulates membrane cationic channel activities to sequester Na^+ into vacuoles or trigger other signaling pathways for salinity tolerance (Sudhakar et al., 2015). However, the exact pathways of Ca^{2+} on balancing ratio of Na^+/K^+ triggered by PA still need to be further confirmed in other plants species in future. Overall, the effect of PA acts indirectly to regulate both beneficial and toxic salt accumulation in plants.

A significant part of the ability of PAs to regulate protective salt ions or sequester Na^+ is likely due to the effect of PA on activities of tonoplast transporters like H^+-ATPase and K^+/Na^+ cation channels. Plasma membrane depolarization triggered by PA was due to Ca^{2+} efflux and H^+ influx which contribute to Ca^{2+} signaling and modulated a variety of transport processes across the plasma membrane under stress (Pottosin et al., 2014). After 1 mM of Spd or Spm was applied to rice plants, less salinity damage was observed in both salt tolerant and sensitive cultivars. The plants treated with PA had increased plasma membrane (PM) bound H^+-ATPase activity (Roy et al., 2005; Roychoudhury et al., 2011). More recently, Spm synthesis single and double mutants (*acl5-1*, *spms-1* and double *acl5-1*/*spms-1*) of Arabidopsis accumulated more Na^+ in salt sensitive plants compared to wild type plants (Alet et al., 2012). Additionally, patch clamp analysis showed inward Na^+ and outward K^+ blocking by exogenously applied PA and this effect was dependent on the charge of PA at the root epidermal and cortical cells of barley (*Hordeum vulgare*) seedlings under salt stress (Zhao et al., 2007). This suggested that PA may have been blocking Na^+ uptake from the roots. In addition, the effect of PA is to sequester Na^+ into vacuole to protect plants from salinity damage. Over-expression or transgenic manipulation of PA biosynthesis pathways in plants to improve plant salinity tolerance was due to increased activity of tonoplast K^+/Na^+ selection and efficient vacuolar Na^+ sequestration from Spd and Spm (Hamamoto et al., 2008). Such result was confirmed by showing the higher sensitivity of Spm and Spd to the tonoplast K^+ selective channels compared with Put (Hamamoto et al., 2008). In pea (*Pisum sativum*), the non-selective ion channel located on mesphyll cell was inhibited by externally applied PA to reduce K^+ efflux for maintaining K^+/Na^+ homeostasis under salt stress (Shabalaa et al., 2007).

These studies demonstrate the role of PA to manipulate the ratio of K^+/Na^+ to obtain normal growth by affecting H^+-ATPase and tonoplast K^+/Na^+ cation channels activities.

Besides the effect of PA affecting salt transport and accumulation, up-regulation of PA content in response to salinity stress is also correlated to induction of antioxidant systems and stress related defense compounds. In species like pea, exogenously applied Spm enhanced fresh weight and protein contents in pods and seeds under osmotic stress condition. This type of salinity stress alleviation was accomplished by the reduction of lipid peroxidation and a significant elevation of total polyphenol, catalase, and superoxide dismutase activities (Radhakrishnan and Lee, 2013; Fariduddin et al., 2014). Up-regulating the activities of catalase, peroxidase, and polyphenol oxidase in cucumber (*Cucumis sativus*) to increase salinity stress tolerance were also achieved by exogenous Spd (Radhakrishnan and Lee, 2014). In addition to promote antioxidant enzyme activity, PAs act as a non-enzymatic agent controlling the balance between reactive oxygen species (ROS) production and scavenging to mediate H_2O_2 signal against salinity stress. Studies conducted in Ginseng (*Panax ginseng*) (Parvin et al., 2014) and Kentucky bluegrass (*Poa pratensis*) (Puyang et al., 2015) showed increased salinity tolerance by enhancing the ROS scavenging enzyme activities. These results showed the effect of PA in mitigating the salt stress by increasing the anti-oxidative enzyme activities.

Drought stress

Drought is another main environmental stress due to water deficit which influences plant growth and development and causes yield loss in crop plants. PA has been implicated in drought stress responses and tolerance. Higher conjugated Spd and Spm levels were observed in leaves of drought tolerant common wheat (*Triticum aestivum*) compared to the sensitive

one under dehydration stress (Liu et al., 2006). In addition, under severe dehydration stress by using polyethylene glycol (PEG) as dehydration agent, free and conjugated Put contents increased and then decreased as increase of PEG concentration. However, under moderate dehydration condition, free and conjugated Spd and Spm in leaves increased for maintaining normal growth osmotic pressure for survival (Zhou et al., 2010), which suggested the protective effect of Spd and Spm from dehydration. Similarly, progressive decrease of free PA while increased cell wall bound PA were observed at tillering, heading, and anthesis stage in triticale under water stress (Hura et al., 2015). Thus, at least for several plant species, a link exists between drought stress tolerance and elevated free or conjugated PA content.

Coupling with the regulation of PA content, manipulation of PA content through transgenic manipulation and exogenous application were used to show the effect of PA upon drought stress. In grape plants, the highest change in free PA, up regulation of *ADC* and their corresponding enzyme activities were found in the drought tolerant genotypes (Hatmi et al., 2015). Based on this correlation of PA content induction and drought tolerance, exogenous PA application has also been found to protect against drought stress. In rice, 10 μM of Spm was identified as the most effective protective agent against drought stress using foliar application (Capell et al., 2004; Farooq et al., 2009). Similarly, results were obtained by exogenously applied PA to alleviate water deficit induced membrane permeability by reducing lipoxygenase activity in cucumber roots (Kubis et al., 2014). In addition, transgenic lines were produced by over expressing *ADC2* and such lines showed drought tolerance by reducing water loss (Alcazar et al., 2010). Stress-responsive elements were found in the promoters of certain genes playing a role in PA synthesis which resulted in an early activation of PA biosynthesis in response to drought stress (Pottosin et al., 2014). This increased content

of PA through various types of approaches has shown association of PA content regulation and drought tolerance.

PA may protect plants from drought stress by signaling and cross-talk with other phytohormones, mediating membrane ion channels, and inducing stress defense related compounds. Experiment conducted by Liu et al. (2000) in wheat under drought stresses indicated PA as signaling molecule to regulate the voltage dependent inward K^+ channel in the plasma membrane of guard cells and modulate stomatal closure for water conservation. In the regulation of stomatal aperture, the plant hormone abscisic acid (ABA) acts as a key regulator of K^+ concentration across guard cell membranes to close stomata to restrict transpiration and water loss under drought stress (Grill and Christmann, 2007). Additionally, PAs act against drought stress in wheat (*Triticum aestivum*) embryo by increasing plasma membrane H^+-ATPase activity by up-regulating the levels of free Spd, Spm, and conjugated Put and Spd (Du et al., 2015). Up-regulation of some other stress responsive elements, hormones, and signaling related genes to combat stresses via PA has also been found. For instance, PA involvement in the biosynthesis of auxin, ethylene, ABA, gibberellins, salicylic acid, auxin transport, auxin-responsive, ethylene and ABA responsive transcriptional factors, and jasmonate-induced proteins were also found (Marco et al., 2011). These results indicate the role of PA against drought stress by interacting with other defense related genes and hormones.

PA oxidation is also involved in drought stress signaling by interacting with other hormones and signaling compounds such as ABA and H_2O_2. H_2O_2 is the byproduct of Spd and Spm oxidation by PAO (Bagni and Tassoni, 2001). In grape plants, PAO enzyme activity increased in drought tolerant plants compared to sensitive ones by maintaining PA

homeostasis for tolerance (Hatmi et al., 2015). Other studies showed the involvement of enzymes specific for other PA oxidation in relation to drought tolerance. An et al. (2008) demonstrated that Put degradation through copper-containing amine oxidases (CuAO) produced increased level of H_2O_2 in broad bean to be drought tolerant compared with the control plants. They also found increased H_2O_2 triggered the increase of Ca^{2+} in the guard cells in response to ABA for stomatal closure (An et al., 2008). These results indicated that H_2O_2 is associated or interacts with ABA signaling pathways to induce stomatal closure. However, PA metabolism may not be involved in drought stress tolerance in all species. In species of cotton (*Gossypium hirsutum*), clear relationship between drought tolerance and PA changes was not established through PA metabolism in both reproductive and vegetative stages due to water deficit stress (Loka et al., 2015)**Error! Reference source not found.**. At molecular level, PA oxidation is regulated by ABA as well. Transcripts of the *MPAO* mRNA level in maize were enhanced by external ABA treatment (Wimalasekera et al., 2011). In addition to oxidation of PA interacting with hormone pathways, PA biosynthesis genes are influenced by ABA signaling. PA biosynthesis genes like *AtADC2*, *AtSPDS1*, and *AtSPDS1* expression levels were down-regulated in ABA-deficient (*aba2-3*) and ABA-insensitive (*abi1-1*) mutants in Arabidopsis (Alcázar et al., 2006). This shows the signaling effect of PA oxidation and influence of phytohormone ABA on the PA oxidation to trigger drought stress tolerance. Additional studies to better understand potential PA involvement in drought stress signaling such as in relation to other hormones or drought stress protective compounds are needed.

Cold stress

Cold stress affects growth and productivity of plants by direct inhibition of metabolic reactions, cold induced osmotic stress, or chilling induced cellular dehydration, which can reduce cellular membrane fluidity, alter the structure of macromolecules, and inhibit enzyme function (Chinnusamy et al., 2007). Acclimation increases chilling tolerance in response to low non-freezing temperatures based on significant changes in gene expression and metabolite profiles. PAs are metabolites involved in plant responses to cold. In order to illustrate the role PA play in response to chilling stress in plants, PA content and its biosynthetic gene expression changes have been investigated. For example, a significant increase in Put content during chilling stress was observed in cucumber (Shen et al., 2000). Put increased in leaves of chilling tolerant tomato compared with the sensitive ones. This Put content increase was correlated to the increase of ADC activity and its gene expression level (Song et al., 2015) which demonstrates the involvement of Put in tomato chilling tolerance. Spd biosynthesis inhibitor of methylglyoxal-bis-(guanylhydrazone), which inhibits Spd accumulation, was applied to cucumber plants and showed enhanced chilling injury compared to the control plants and this effect was reversed by simultaneous Spd treatment (Shen et al., 2000). Additionally, up regulation of PA biosynthesis genes in response to cold acclimation was investigated. Mo and Pua (Mo et al., 2002) showed Put biosynthesis cDNAs of *MADC2-3* in mustard (*Brassica juncea*) were stimulated under chilling stress. Likewise, PA biosynthesis genes, *ADC1*-2 in Arabidopsis (Cuevas et al., 2008; Alcazar et al., 2011), *PaADC1*-2 in Pringlea (*Pringlea antiscorbutica*) (Hummel et al., 2004), and *MfSAMS1* in alfalfa (*Medicago falcata*) (Guo et al., 2014) were all up-regulated in response to cold stress,

which suggests the positive correlation of cold stress tolerance and PA content up regulation owing to their biosynthetic gene expression.

PAs have potential effects on membrane lipid profiles during cold stress incidence to improve cold tolerance. Membrane lipid damage by cold stress can cause accumulation of ROS to cause further plant physiological damage. Fatty acid desaturases were found to contribute to the mediation of unsaturated fatty acid level to change membrane fluidity in response to abiotic stresses (Upchurch et al., 2008). Linoleic acid (18:2) showed accumulation in the cold acclimated potato (*Solanum commersonii*) compared with the non-cold acclimated cultivars which suggested the importance of de-saturation in cold tolerance (Vega et al., 2004). Furthermore, coupling with this de-saturation activity, transcription level of the catalyzing enzyme stearoyl-ACP (Δ^9) desaturase was also higher than in the acclimated compared with the non-cold acclimated species (Vega et al., 2004). However, how PA is involved in the fatty acid composition changes to improve chilling stress tolerance was investigated. A study conducted on metabolite profiling analysis showed negative relationship between Spd and trans-Δ^3-hexadecanoic acid (16:1) in phosphatidylglycerol fraction in response to cold stress in different cereal (wheat, barley, and oat) genotypes (Gondor et al., 2016). Besides, sucrose pretreatment in banana tissue culture enhanced its cold stress survival by increasing Put content and ratio of unsaturated/saturated fatty acid which indicated the communication between Put and fatty acid for improving cold tolerance (Ramon et al., 2002). These studies point out that lipid fraction changes in thylakoid membrane triggered by PA may have played a role in cold tolerance.

Often under cold stress, plants may experience cellular water loss or drought stress-like symptoms. Thus, much like during drought stress, PAs exhibit interaction with ABA to

promote cold tolerance. Mutation or inhibition of PA biosynthesis genes has been done to confirm whether cold stress sensitivity is affected. Put mutants of *adc1*-2 in Arabidopsis suffered detrimental effects under cold treatment (Cuevas et al., 2008). In this mutant, reduction in ABA levels and expression of 9-cis-epoxycarotenoid dioxygenase (*NCED3*) for ABA biosynthesis was also observed. However, such detrimental effect was complemented by ABA and normal Put production which suggested that Put functions against cold stress was ABA-dependent signaling pathways (Cuevas et al., 2008). Additionally, induction of nitric oxide synthesis through PA (Spd and Spm) catabolism by CuAO and PAO could improve stress tolerance via its signaling function which indicated the interaction complex against cold stress due to PA production (Wimalasekera et al., 2011). However, this area has not been thoroughly investigated which might serve for future study.

Heat stress

Heat is another type of non-optimal temperature growing condition that causes damage to membranes, inhibition of enzymatic reactions, and other stress damages (Pospíšilová, 2007). Heat stress-induced growth and development inhibition also largely can be a rapid induction of ROS and reduced carbon assimilation. PA can exhibit dynamic changes in response to heat stress in different plant species or due to differences in stress severity. For instance, free and conjugated Put increased after heat stress while free Spd, norspermidine, and Spm accumulated with two hours lag and such PA induction was correlated to PA biosynthetic enzyme induction (Cvikrova et al., 2012). Conversely, some plants exhibit opposite response in PA content during heat stress by accumulating lower endogenous PA content when exposed to stress. Similarly, opposite results in PA content profile shifts in response to heat stress were obtained among twelve rice cultivars with different high night temperature

sensitivities. Plants that were heat tolerant accumulated less PA and increased expression of *ACD2* was only restricted in the sensitive cultivars (Glaubitz et al., 2015). The PA biosynthesis gene expression is also dynamic to heat stress. In Arabidopsis, Spm synthase genes *SPMS* and *SAMDC2* were induced in the early stages of heat stress and persistent heat stress resulted in induction of *ADC2* (Sagor et al., 2013). Since PA content changes may be significantly affected by many environmental or experimental variables and plant species, determination and interpretation of the effects on PA biosynthesis and genes expression in response to heat stress cannot be generalized.

PA biosynthesis genes have also been found to trigger heat shock proteins induction and other heat protective proteins such as antioxidant enzymes. Both exogenous application of Spm and over-expression of *SPMS* promoted heat stress tolerance in Arabidopsis and heat shock proteins (HSP) of HSP101, HSP90, HSP70, and HSP17.6 were activated by coupling with the heat shock (Sagor et al., 2013). Furthermore, antioxidant enzyme activity was found to be enhanced during heat stress-induced PA regulation. Transgenic tomato harboring over-expressed *SAMDC* gene showed higher amounts of Spm production and thermotolerance than the wild type by enhancing antioxidant enzyme activity (Cheng et al., 2009). Although Spm is considered to be the most effective PA against heat stress, further supportive experiments by transgenic and exogenously applied Spm alleviating heat stress might be very helpful for better interpretation the role of PA in heat stress.

Heavy metals

Environmental pollution or commercial mining sites may cause soil to have heavy metal contamination causing phytotoxicity to plants due to bio-availability through plant roots. Such phytotoxicity in plants is caused by induced ROS production, enzyme cofactors or

transcription factor replacement, cellular redox and ionic transport imbalance, DNA damage, protein oxidation, or other stress damages (Sytar et al., 2013; Huang et al., 2012). Due to heavy metal stress, plants can be stunted in growth and have reduced chlorophyll content, inhibited photosynthesis, and restricted reproductive potential (Gill et al., 2010). It is apparent that PAs may play a role in plant tolerance of certain heavy metals through several mechanisms. Cadmium (Cd) stress can readily occur even at relatively low concentrations since it is readily and rapidly taken up by plant roots (Groppa et al., 2001). Cd^{2+} and Cu^{2+} induced heavy metal stress was recovered by exogenously applied PA by increasing the antioxidant activities in sunflower (*Helianthus*) discs (Groppa et al., 2001). However, they did not find significant induction of all three PAs due to Cd^{2+} and Cu^{2+} stress (Groppa et al., 2001). Increased free and conjugated PA concentration via Cd application to cultivar bright yellow 2 (BY-2) of tobacco plant leaf (*Nicotiana tabacum*) was found to combat such stress. This stress tolerance was due to maintenance of PA homeostasis to stabilize membrane stability and scavenge free radicals (Kuthanova et al., 2004). Due to this increased content of PA in response to heavy metal stresses, the role of PA play in heavy metal tolerance has been evaluated by exogenous application of PA. For instance, applied Put and Spd lead to phytoremediation which worked as Ni chelator for detoxification in Amaranthus (*Amaranthus paniculatus*) leaves (Shevyakova et al., 2011). Additionally, over-expressed Spd synthase gene in European pear (*Pyrus communis*) showed heavy metal tolerance by exerting increased antioxidant activities (Wen et al., 2010). More research is needed in order to better understand the effects of PA on heavy metal tolerance and exploitation of PA pathways may be useful within phytoremediation technologies.

Other abiotic stresses

Mechanical or insect herbivory can cause wounding to plants which triggers a series of signal transduction events. Relative to abiotic stress studies, fewer studies have been conducted on investigating PA interactions with other internal signals during mechanical wounding. However, a few studies show decreased Spm while increased Put content accompanied by *ADC2* gene expression as response to wounding in Arabidopsis (Perez-Amador et al., 2002) and oilseed rape (*Brassica napus*) (Cowley et al., 2005). Similarly, Put and Spd were found to be induced due to wounding in potato (*Solanum tuberosum*) tubers which also corresponded to the mRNA up regulation of *ADC*, *ODC*, and *SAMDC* (Lulai et al., 2015). In addition, *ADC2* mRNA was up-regulated in the jasmonic acid (JA) insensitive *coi1* mutant, which indicated dual regulation of *ADC2* by JA signaling pathways in responses to wounding (Perez-Amador et al., 2002). Furthermore, local and systemic increase of CuAO/DAO in wounded chickpea (*Cicer arietinum*) indicated the role of CuAO/DAO for H_2O_2 production in response to wounding (Rea et al., 2002). Studies showed more profound effect of other phytohormones and physiological relevance of PA involved in wounding responses might fill the gap of this part of research in future.

In addition to mechanical wounding, leaf tissue can be damaged due to ultraviolet (UV) radiation. Due to stratospheric ozone layer depletion, anthropogenic pollutants, or natural causes, UV radiation can stress plants by causing damage to DNA, proteins, lipids, and membranes (Rowland, 2006; Hollosy, 2002). PAs have been shown to be involved in plant responses to UV light (Hollosy, 2002). In Arabidopsis, *ADC* and *SPMS* were up-regulated under low to medium UV-B (280-320 nm) radiation compared with the chronic UV-B acclimated plants, which did not affect the expression of stress responsive genes (Hectors et

al., 2007). In addition, Put and Spm were found to regulate the amount of light energy reaching the reaction centers to influence the sensitivity of the photosynthetic apparatus from damage by exposing the plants under different levels of UV radiation (Sfichi et al., 2004; Lütza et al., 2005). Additionally, the protective effect of Spm against UV-C (200-280 nm) exposure was demonstrated by external application of Spm to pea plants. The plants treated with Spm maintained normal plant growth, stabilized cell membranes, and activated non-enzymatic antioxidants (Todorova et al., 2013). More recent research is still needed to elucidate the role of PAs in UV radiation defense.

Conclusions and future perspectives

Major effects of PA in abiotic stress tolerance are largely through direct or indirect stress signaling, membrane health maintenance and mediation of ionic transport and antioxidant systems, as well as other defense mechanisms. PA content and biosynthesis gene expression can vary based on abiotic stress type, stress severity, plant species, or even cultivar. Regardless of which PA form may be more important for a given abiotic stress or species, each PA seems to play an important role in stress survival. Research to specifically evaluate the utility of PA in a wide range of agricultural plants is needed. It seems that the majority of the work on PAs is still primarily focused on model plant species. For instance, the effects of exogenous application of PAs or manipulation of PA pathways should be more rigorously compared against other compounds that are also shown to improve stress tolerance, such as proline, abscisic acid, and many others. Are PAs more effective than these compounds or less? Are PAs in combination with other stress protective compounds beneficial? Answering such questions is important to determine whether PAs are important to exploit in applied agricultural setting or in plant breeding. Further research on investigating the relationship

between PAs and other abiotic stress tolerant genes, hormone responses, and other major processes such as photosynthesis and respiration is needed. Additionally, more detailed studies using next generation sequencing and other new technologies that are able to identify specific changes in gene expression and metabolic responses to altered PA accumulation or regulation are warranted. What's more, how PAs may play a role in stress signaling, leaf senescence, programmed cell death and other processes shared by many abiotic stresses will help elucidate the role of PA in stress tolerance. Whether PA transport, localization, and allocation throughout plants during stress conditions plays a role in stress sensing and defense also may deserve further investigation.

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CHAPTER 2

POLYAMINE CONTENT CHANGES IN CREEPING BENTGRASS EXPOSED TO SALT STRESS

Abstract

Salt stress is a major problem in turfgrass management. Investigation of metabolites, such as polyamines (PAs), that may improve salt tolerance of turfgrass species is needed. Two independent growth chamber studies were conducted to evaluate physiological characteristics and changes in PAs, such as putrescine (Put), spermidine (Spd), and spermine (Spm), in response to salt stress in 'Penncross' and 'PsgSLTZ' creeping bentgrass (*Agrostis stolonifera*). The study also aimed to determine a method of PA extraction to improve PA yields from creeping bentgrass. Salt solutions were drench applied to plants growing in pure sand daily in a stepwise fashion for approximately 70 d in both studies. For both cultivars, salt stress caused an increase in leaf Na^+ content, percent of electrolyte leakage, and canopy temperature depression while it caused a decrease in turf quality, osmotic potential, and K^+ and Ca^{2+} content compared to controls. In the early stages of salt stress, Put content increased in salt stressed plants compared with controls. Spd content did not change significantly while a transient increase in Spm was observed in the later stage of salt stress. The PA quantification method used in this study included using formic acid during the extraction process, which exhibited enhanced quantification of PAs from creeping bentgrass compared to other methods previously published. Salinity stress up-regulated the content of Put and Spm in leaf tissue which may be involved in salinity tolerance in creeping bentgrass while Spd accumulation may not be a major salt tolerance mechanism; supplementation with these biochemical compounds could be an alternative to improve creeping bentgrass salt tolerance.

Key words: Salt stress, creeping bentgrass, putrescine, spermidine, spermine

Introduction

Salt stress is a major issue for turfgrass management around the world. Use of reclaimed, non-potable water that may contain high levels of salts is becoming a common management practice (Duncan et al., 2008). Additionally, numerous turf areas are located in salt-prone environments, such as shorelines or areas with salt-afflicted soils. The primary damage to plants caused by salinity is due to toxic ion accumulation of Na^+ in plant tissues and water deprivation from decreased external osmotic potential in the soil solution (Hasegawa et al., 2000). Creeping bentgrass does not possess major salt resistance mechanisms to rid plants of salt, such as salt glands, that are found in other grass species within Poaeceae (Marcum, 2001). Since adequate avoidance mechanisms are not possessed by creeping bentgrass, the species must rely on other salt tolerance mechanisms for survival under salt stress conditions. Evaluating mechanisms that may promote tolerance to salt stress is necessary to development of salt tolerant creeping bentgrass germplasm. Cultivar variation in salt tolerance of creeping bentgrass does exist, but, the major metabolite pathways that may play a role in differential salt tolerance mechanisms between cultivars have not yet been fully elucidated. Additionally, knowledge of plant metabolites important in salt stress tolerance could lead to the development of new plant protective chemical products.

A relatively unexplored biochemical process in turfgrass species is the PA biosynthetic pathway. The common PAs that associated with growth regulation and abiotic stresses are: putrescine (Put), spermidine (Spd), and spermine (Spm). PAs are synthesized from arginine or ornithine decarboxylation pathways (Janowitz et al., 2003). PAs may respond differently to abiotic stress based on plant species, plant tissue type, and

developmental stage (Capell et al., 2004). In Arabidopsis (*Arabidopsis thaliana*), there was a shift in PA biosynthesis pathways toward accumulating Put whereas in the resurrection plant (*Craterostigma plantagineum*) this shift was toward accumulating Spm (Alcazar et al., 2011). Conversely, after salt stress in broad bean (*Vicia faba*) (Sadak and Abdelhamid, 2015) and foxtail millet (*Setaria italica*) (Sudhakar et al., 2015), all three types of endogenous PA concentration increased significantly compared with the control. In a salinity tolerant rice (*Oryza sativa*) cultivar, Put and Spm increased more compared to the sensitive one (Do et al., 2014). In maize (*Zea mays*) leaf blade elongation zone (Rodriguez et al., 2009), an increase of Spd content was observed in response to salt treatment. Recently, Li et al. (2015a) found that PAs were generally up-regulated in white clover (*Trifolium repens*) under artificially induced drought stress in hydroponic cultures. In creeping bentgrass, Spd content decreased due to drought stress, whereas Put and Spm content were increased compared with control plants (Li et al., 2015b). All these results show the importance of PA in salt stress responses. However, it is not yet fully clear whether differences in PA fluctuation during stress is due to species differences, plant developmental stage, or due to difficulties in comparing across studies.

Compared with other crop species, little information is available regarding the function, content, or regulation of PAs in creeping bentgrass or other cool-season turfgrass species, particularly during long term salt stress conditions. For utility as an agricultural technology, determining how the common PAs, such as Put, Spd, and Spm, may respond to stress or play a role in turfgrass tolerance to stress deserves investigation. In addition, current PA quantification methods utilized in other crop species and other turfgrasses did not prove to provide adequate recovery of free PAs from creeping bentgrass tissue in our preliminary tests. Therefore, the objectives of the study were to improve the methods of extraction and

quantification of PAs from creeping bentgrass tissues, evaluate creeping bentgrass physiological health under salt stress, and to quantify PAs from creeping bentgrass in order to achieve a better understanding of PA content regulation due to prolonged salt stress conditions.

Materials and methods

Plant preparation and stress treatment

Two independent growth chamber studies (Expts.1 and 2) were conducted. For both experiments, two types of creeping bentgrass cultivar PsgSLTZ and Penncross were used. Seeds of both cultivars were obtained from the Seed Research of Oregon (Tangent, OR). These cultivars showed good performance under salt conditions in their breeding efforts; however, 'PsgSLTZ' rated higher in salt tolerance based on the seed source information when we purchased. All experimental materials were obtained by seeding at a rate of 4.88 $g \cdot m^{-2}$ in a 12 cm^2 pot with pure sand. Expt.1 was seeded on 18 Aug. 2013 and expt.2 on 14 Mar. 2014. The seedlings for both experiments were established in the greenhouse under 900 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetically active radiation (*PAR*) for 14 h of day length under 65% to 75% humidity condition with 24 ºC/18 ºC (day/night) temperature for approximately two months to attain dense and healthy turf canopy. Then, the fully established seedlings were transferred to an environmentally controlled growth chamber with 700 $\mu mol \cdot m^{-2} \cdot s^{-1}$ *PAR* for 14 h of day length under 65% to 70% humidity condition with 23 ºC/18 ºC (day/night) temperature for both experiments.

Plants to be stressed by salt solution were placed in a randomized complete block design with four biological replications. All salt treated plants were watered daily each week with100 mL of salt solution at 0 (21 Dec. 2013), 12 (7 d), 12 (21 d), 16 (35 d), 16 (42 d), 20

(56 d), and 24 (70 d) $dS \cdot m^{-1}$ for expt. 1. The salt solution (Instant Ocean Aquarium Salt, Blacksburg, VA) was made with half strength Hoagland solution (Hoagland and Arnon, 1950) as nutrient supplement twice a week while salt solution was made from de-ionized water for the rest of the 5 d within the week. The salt solution included 10.78 $g \cdot L^{-1}$ Na^{+}, 0.42 $g \cdot L^{-1}$ K^{+}, 1.32 $g \cdot L^{-1}$ Mg^{2+}, 0.4 $g \cdot L^{-1}$ Ca^{2+}, 0.008 $g \cdot L^{-1}$ Sr^{2+}, 19.29 $g \cdot L^{-1}$ Cl^{-}, 2.66 $g \cdot L^{-1}$ SO_4^{2-}, 0.2 $g \cdot L^{-1}$ HCO_3^{-}, 0.56 $g \cdot L^{-1}$ Br^{-}, and 0.001 $g \cdot L^{-1}$ F^{-}. The percent of NaCl in the salt solution was 84%. In order to obtain long term effect of salt stress, the same amount of salt solution as used in expt. 1 were applied at each time point using 0 (6 May 2014), 4 (28 d), 8 (35 d), 12 (49 d), 16 (63 d), and 20 (77 d) $dS \cdot m^{-1}$ for expt. 2.

Physiological traits evaluation

Turf quality (TQ) rating was based on color, density, and uniformity of the grass canopy using a scale of 1-9 with 1 being dead plants and 9 being healthy and green plants (Beard, 2001). Percent electrolyte leakage (EL) was measured to estimate cell membrane stability of leaves after salt treatment based on the method of Blum and Ebercon (1981). Approximately, 10 leaves were cut, briefly washed, and immersed in 15-mL falcon tube with 7.5 mL of de-ionized water and placed on a shaker for 24 h at 144 rpm. The electric conductivity of the solution containing the living tissue was measured using electric conductivity meter (3200 conductivity meter, YSI, Yellow Springs, OH) as initial conductivity (Ci) after shaking. The leaf tissues were completely damaged by boiling them in a water bath for 5 h and then were placed on a shaker with the same speed for another 24 h. The electric conductivity of the solution containing dead tissues was measured as the maximum conductivity (C_{max}). The percent EL was calculated as $C_i/C_{max} \cdot 100$.

Osmotic potential (Ψ_s) was determined similar to the method in Qian and Fry (1997). Approximately 10 leaves taken from each plant were immersed in de-ionized water in a petri dish for 3 h and leaves were blot dried and transferred into a 2-mL micro-centrifuge tube and immediately frozen in liquid nitrogen and stored at -80 °C. Samples were ground with a micro-pestle and centrifuged at 20,817 x g (Eppendorf 5430 R, Scientific, Inc. Ocala, FL) for 15 min under room temperature. 10 µL of the leaf extract was placed on a piece of 0.5 cm diameter filter paper, inserted into a vapour pressure osmometer (5520 VAPRO; Wescor, Inc., Logan, UT) to obtain leaf solute molarity. The conversion from molarity to MPa was made as $\Psi_s = - C \cdot R \cdot T$ where C is the leaf solute molarity, T is the absolute temperature of 310 K, and R is the constant (8.314 $cm^3\ MPa \cdot K^{-1} \cdot mol^{-1}$).

Canopy temperature depression (CTD) is an indication of transpiration rates and has been widely used as a method to evaluate plant stress (Blum et al., 1982). The turf canopy temperature was measured using an infrared thermometer (Davis Instruments; Vernon Hills, IL). The temperature depression was calculated by subtracting ambient temperature in the growth chamber from the plant canopy temperature.

Mineral nutrient determination

For both experiments, one gram of grass leaf tissues was harvested and dried at 60 ^oC in an oven for 3 d and were ground through a 1-mm screen within a stainless steel grinder (Wiley Mini-Mill Stainless Steel, 120V, 60 Hz; Thomas Scientific; Swedesboro, NJ) (Plank, 1992). The same amount of ground leaf tissue was digested in 10 mL 16 N of HNO_3 for 30 min in a microwave and the prepared sample volume was brought to 50 mL with de-ionized water prior to measurement (Jones, 2001). Na^+, K^+, and Ca^{2+} were measured using inductively coupled plasma (ICP)-mass spectrometer (MS) (Optima 3300 DV ICP-MS emission

spectrophotometer; A & L Great Lakes Laboratories, Inc. Fort Wayne, IN) at wavelengths of 589.592, 766.490, and 317.933 nm, respectively. Final results were reported as $mg \cdot g^{-1}$ DW.

Polyamine extraction

The method of Liu et al. (2011) and Oefner et al. (1992) was used with modification to quantify the accumulation of free PAs in creeping bentgrass for both experiments with four biological replications. Modifications were necessary to improve yield of PA from creeping bentgrass samples in our preliminary studies. The major modification included use of 5% formic acid (Sigma-Aldrich, St. Louis, MO) during PA extraction instead of perchloric acid. For PA extraction, a 250 mg sample of frozen leaf tissue was ground with liquid nitrogen to obtain a fine powder with a mortar and pestle. An aliquot of 1 mL of 5% formic acid (prepared with water) was added to the ground leaf powder and they were ground further until fine slurry was reached. Ground sample slurry was collected in 2-mL micro-centrifuge tube and stored in -80 ^{o}C for further processing. In order to prepare the samples for derivatization, the samples were allowed to thaw at room temperature and centrifuged for 30 min with 20,817 x g at 4 ^{o}C. The supernatant containing PAs was transferred to a fresh sterile 2-mL micro-centrifuge tube. The pH of the supernatant was adjusted to be higher than 12.0 with 2M NaOH (Fisher Scientific, Fair Lawn, NJ). The samples were then dried in a vacuum evaporator (79703-00 centrivac; Labconco, Kansas City, MO) at room temperature.

The dried samples were re-suspended in 500 µL of 4% benzoyl chloride (prepared in acetone; Sigma-Aldrich) and incubated at 30 ^{o}C for 2 h with shaking. After incubation, the supernatant was taken after centrifugation (20,817 x g) and was mixed with 500 µL of saturated NaCl solution and 1 mL of dichloromethane (99%; ACRO, Bridgewater, NJ) in a 15-mL centrifuge tube. Samples were centrifuged at 20,817 x g for 5 min at room temperature.

Following centrifugation, the bottom layer containing derivatized PAs was collected with a pipette. Benzoyl chloride derivatized PAs in dichloromethane (total 2 mL volume) were washed with an equal amount of water three times to get rid of salts by pipetting. Finally, samples were lyophilized in a freeze dryer (Genesis Pilot, Genesis 25L, SP Scientific, Warminster, PA) at room temperature. Dried samples were re-suspended in 60% methanol for high performance liquid chromatography mass spectrometry (HPLC-MS) analysis.

External standard preparation

Put, Spd, and Spm (Sigma-Aldrich) stock solutions of 0.5 $mM \cdot L^{-1}$ were prepared in 5% formic acid. Samples were evaporated to dryness at room temperature in vacuum concentrator (Savant SPD111V, Thermo Fisher Scientific, Waltham, MA) followed by benzoyl chloride derivatization as described above. Finally, standards were re-suspended in 60% methanol to prepare standard curve just before loading onto the LC-MS mass spectrometry as same as the other analytes described below. The positive ions for detection of Put, Spd, and Spm were m/z 319.1, m/z 480.2, and m/z 641.3, respectively. The reproducibility of the instrument was validated by injection the mixtures of those three standards at the concentration of 30 $\mu M \cdot L^{-1}$. A 60% methanol solvent was used as a blank between every six analytes to show no carry over from previous sample.

Instrumentation and chromatographic conditions

Both experiments were performed at room temperature based on Liu et al. (2011) with modifications. Benzoyl chloride derivatized PA samples were analyzed using LC-MS mass spectrometry (Quattro micro; Waters, Inc., Milford, MA) coupled with a binary pump (LC-20 AD) and auto-sampler (SIL 5000 Auto injector, Shimadzu Scientific Instruments Shimadzu Corporation, Columbia, MD). Derivatized PAs were injected into a C18 column (5 cm X 2.1

mm X 2.7 μm; Ascentis Express, Sigma Aldrich) at the gradient of 30% phase B and 70% phase A for the first 5 min, 74% B and 26% A for the next 10 min; 30% B and 70% A started at 10.01 min and was kept for another 3 min to get column equilibrium with the flow rate of 0.3 $mL \cdot min^{-1}$. The mobile phase A and B are water and methanol, respectively. The electro spray ionization positive mode and SIR (single ion recording) data type were used to acquire PAs Na adduct ions. The source and dissolution temperature was set at 100 ^{o}C and 350 ^{o}C, respectively. Capillary and cone voltage was 3.17 kV and 35 volts, respectively. Dissolution gas flow was set at 600 $L \cdot h^{-1}$ and cone gas flow was 30 $L \cdot h^{-1}$.

Experimental design and statistical analysis

Initially, all data of each trait were tested for their normality and homogeneity using the univariate procedure in SAS (version 9.4; SAS Institute, Cary, NC). All data were normally distributed with uniform homogeneity. Therefore, all of the raw data were analyzed by using PROC MIXED based on the mixed linear model in SAS. Significant differences ($P \leq 0.05$) of means for each trait at each time point were separated by using Fisher's least significant difference (LSD) test in the lsmeans procedure.

Results

Physiological traits evaluation

In both experiments, TQ declined due to salt stress whereas control plants did not decrease in TQ (Figure 1). TQ decreased significantly after approximately 42 d (16 $dS \cdot m^{-1}$) of salt treatment in expt. 1 (Figure 1A). However, in expt. 2, the quality decreased in 'PsgSLTZ' was slower than 'Penncross' which happened after 49 d (12 $dS \cdot m^{-1}$) of salt treatment (Figure 1B). Major differences in TQ were detected when comparing cultivars under salt treatment on two dates (35 and 70 d) in expt. 1 (Figure 1A). EL remained consistently low at approximately 12%

for control plants in both experiments (Figure 1C and D). In both experiments, EL levels began to significantly increase in salt treated plants compared to controls of both cultivars at 42 d (16 $dS \cdot m^{-1}$) and 49 d (12 $dS \cdot m^{-1}$) of salt treatment (Figure 1C and D). EL level of 'PsgSLTZ' generally was higher than in 'Penncross' in the later stage of salt treatment in both experiments (Figure 1C and D). Ψ_s levels in both cultivars were maintained at approximately -0.5 MPa for control plants throughout the duration of both experiments (Figure 2A and B). Approximately, 28 d after salt treatment, a significant decrease in Ψ_s was observed in salt treated plants from both experiments and this trends was followed by prolonged salt stress as well (Figure 2A and B). However, the amount of decrease in 'PsgSLTZ' was significantly ($P \leq 0.05$) less than in 'Penncross' on some sampling days in both experiments (Figure 2A and B). No significant differences were detected for CTD between cultivars in both experiments under both control and salt stressed conditions (Figure 2C and D). However, control plants exhibited significantly lower CTD than the salt-stressed plants in both experiments (Figure 2C and D). In addition, 'PsgSLTZ' exhibited slightly higher CTD compared with 'Penncross' in both experiments (Figure 2C and D).

Mineral nutrient content

Plant leaf Na^+ content increased significantly due to salt treatment in both cultivars of both experiments (Figure 3). Under moderate salt stress condition (21 d), both cultivars had similar Na^+ content accumulation in both experiments (Figure 3A and B). After prolonged salt stress (70 d), 'PsgSLTZ' accumulated significantly less Na^+ than 'Penncross' from both experiments (Figure 3A and B). Plant leaf Ca^{2+} content decreased significantly compared to controls for both cultivars due to salt stress in both experiments (Figure 3C and D). No significant differences were detected due to salt stress in Ca^{2+} content change between two cultivar types

from two experiments (Figure 3C and D). Plant leaf K^+ content decreased significantly due to salt stress in both experiments (Figure 3E and F). 'PsgSLTZ' had significantly higher K^+ content than 'Penncross' on 35 and 70 d of salt stress in expt. 1 (Figure 3E) and on days of 49, 63, and 77 in expt. 2 (Figure 3F).

PA content

Before optimizing extraction, column size, solvent and mobile phase described hereby, no peak separation for Spd and Spm were detected. The area percent after optimizing the method was 94% for three PAs standards. PA extraction in formic acid instead of perchloric acid proved to be a more effective method for extraction of PAs in LC-MS detection from creeping bentgrass tissues. The method was validated by using all three standards calibration curve from fixed concentration of 0.1, 1, 2, 5, 10, 25 $\mu M \cdot L^{-1}$ and the linearity correlation coefficient (r^2) between peak area and concentration for Put, Spd, and Spm as 0.983, 0.984, and 0.971, respectively. The instrument carry over was not detected based on no peaks at the designated retention time points by placing 60% of methanol between every six analytes and the standard deviations for all the analytes ranged from 0.0% to 0.5%.

Based on each linear curve of the PA standards, the range of quantities of the sample ranged from 263 $nmol \cdot g^{-1}$ FW to 5607 $nmol \cdot g^{-1}$ FW. Salt stress caused an increase in Put production compared to control plants in both experiments (Figure 4A and B). However, Put content induction in 'PsgSLTZ' was lower compared to 'Penncross' on 7 and 21 d after salt treatment in expt. 1 (Figure 4A). In expt. 2, it showed similar trend except for 28 d after salt treatment in which 'PsgSLTZ' had high Put content compared to 'Penncross' (Figure 4B). The overall content of Spd production was greater in expt. 1 (Figure 4C) compared to Expt. 2 (Figure 4D). Spd content in 'PsgSLTZ' was significantly higher compared to 'Penncross' in

the early stage (21 d) of salt stress in expt. 1 (Figure 4C). However, for the remaining time points measured for Spd, no significant differences were detected in salt treated plants compared with the control plants from both experiments (Figure 4C and D). Spm production was generally induced by salt stress in both experiments (Figure 4E and F). For expt. 1, Spm content was significantly greater in salt treated plants on 21 and 35 d (Figure 4E). On these days, 'PsgSLTZ' had significantly greater Spm content than in 'Penncross' (Figure 4E). Under prolonged salt stress conditions (70 d), Spm content was at or below the level of control plants (Figure 4E). A similar trend was observed for Expt. 2 (Figure 4F). On 49 d of treatment, 'PsgSLTZ' had a greater level of Spm content whereas on 63 d of treatment 'Penncross' had significantly greater levels of Spm (Figure 4F).

Discussion

Physiological traits evaluation results in the study indicate that both 'PsgSLTZ' and 'Penncross' creeping bentgrass experienced similar levels of damage due to salt treatments in both experiments. Here we found that salt exposure of these two creeping bentgrass cultivars resulted in an accumulation of Na^+ in plant leaves and a reduction in Ca^{2+} and K^+ content in both experiments. 'PsgSLTZ' exhibited significantly higher K^+ (throughout salt treatment), significantly lower Na^+ (on the last day of salt stress in both experiments), less change in Ψ_s (on most dates of both experiments) compared to 'Penncross' in both experiments. This is relatively consistent with our previous studies of other creeping bentgrass cultivars differing in salt tolerance (Krishnan and Merewitz, 2015). Salt stress has shown to reduce the content of K^+ and Ca^{2+} and maintenance of K^+ is associated with salt tolerance (Krishnan and Merewitz, 2015; Qian and Fu, 2005; Sairam et al., 2002). However, overall we cannot fully conclude whether the cultivars differed in salt tolerance from the results of this study, as more

morphological and physiological data would be required. Further research and physiological characterization is needed to better understand potential salt tolerance attributes of various creeping bentgrass cultivars.

Creeping bentgrass does not readily exhibit salt escape or avoidance mechanisms (Marcum, 2001). Plants could utilize salt tolerance mechanisms such as accumulation of osmolytes, production of antioxidants, and other mechanisms to deal with salt accumulation (Fry and Huang, 2004). A major tolerance mechanism to various abiotic stresses in creeping bentgrass is proposed to be the regulation of gene expression and biochemical pathways controlling PA content homeostasis. Common PAs (Put, Spd, and Spm) are thought to act as regulatory molecules in plant cells by binding and modulating nucleic acids under abiotic stresses (Gill and Tuteja, 2010; Marco et al., 2011; Yang et al., 2007). Some research focuses on the function of conjugated PAs for protection in salt stressed tissues (Quinet et al., 2010; Yang et al., 2007); however, free PA is still the main form of this compound studied for its function under stress conditions. Free PA homeostasis depends on their synthesis, transport, degradation, and conjugation which can be highly complex related to abiotic stress tolerance (Groppa and Benavides, 2008; Minocha et al., 2014). Whether PA content production may be a tolerance mechanism to abiotic stresses in turfgrass species has not yet been fully elucidated. Particularly, little information is available on the function, content, or regulation of these PAs in turfgrass species, such as high value creeping bentgrass.

In order to quantify PA in creeping bentgrass, current methods of PA quantification we used did not work well in our preliminary tests. The method we used here is an economic and simple technique that was modified based on Liu et al. (2011) and Oefner et al. (1992). After optimizing the method, 5% formic acid was used to extract free PA from creeping

bentgrass while Liu et al. (2011) used 10% $HClO_4$ as extraction buffer from human urine. This modification is more accommodating for LC-MS equipment due to its higher volatility compared with perchloric acid. Using LC-MS through electro-spray ionization positive mode and single ion recording (SIR) data type based on the mass to charge ratio of the compound is different from what has been used in other experiments (Li et al., 2015a, 2015b). These experiments have used the method as in Duan et al. (2008) and it is based on UV detection within HPLC (Liu et al., 2007). Comparing to an UV detector, the massspectrometer detection technique in LC-MS is considered as a more precise and free of background interference to quantify PA (Holcapek et al., 2012; Mayr and Schieberle, 2012) because of polarities of PAs (Put^{2+}, Spd^{3+}, and Spm^{4+}; Vafaeezadeh et al., 2016). Furthermore, the retention time (24 min) that Liu et al. (2011) using liquid chromatography quadruple time of flight mass spectrometry (LC/Q-TOF-MS) was longer while our LC-MS used only 13 minutes for PA detection without carryover which is more economical by saving analytic time. Besides, the mobile phase program for their method was 55% A (water) from 0 to 14 min and 74% A from 14 to 24 min which was different from ours (Material and Method). Our mobile phase program is optimized to be beneficial for the mass analyzer to separate and detect PA ions. The flow rate which affects the sensitivity of mass detector in this method (0.3 $mL \cdot min^{-1}$) is different from Liu et al. (2011) (1 $mL \cdot min^{-1}$). Also, smaller column size compared with Liu et al. (2011) is better for higher peak resolution. It is necessary to optimize method as improvement of technologies. To our knowledge only one other published study has evaluated PAs from creeping bentgrass ('Penn-A4') leaf tissue due to exogenously applied PA in response to drought and we have detected approximately 100 times the level of PAs in response to salinity

stress (Li et al., 2015b). However, it is worthy to note that this difference could also be related to differences in cultivar and experimental conditions.

In this study, we have found that the monocot, creeping bentgrass, may exhibit PA regulation in response to salt stress. An increase in Put, no significant changes in Spd, and an increase in Spm were observed due to salt stress of both creeping bentgrass cultivars (Figure 4). Similarly, in grape (*Vitis vinifera*) seedlings, Put and Spm coupled with their biosynthesis genes were induced when grown in media with 200 mM NaCl (Liu et al., 2011). Creeping bentgrass cultivar PsgSLTZ overall exhibited less production of Put and higher production of Spm in response to salt stress (Figure 4A and E) which could be associated with a greater salt tolerance. Zapata et al. (2004) studied free PA accumulation in seven crop species including spinach (*Spinacia oleracea)*, lettuce (*Lactuca sativa)*, tomato (*Solanum lycopersicum*), melon (*Cucumis melo*), pepper (*Capsicum annuum*), broccoli (*Brassica oleraceae*), and beetroot (*Beta vulgaris*) under salinity stress. Slightly alternate results were observed in their study since, in most of these dicot species under salt stress, a decrease in Put and an increase in Spd and Spm was observed. They attributed a higher amount of Spd and Spm with a low amount of Put associating with salt tolerance. A similar trend was also found in seedlings of common wheat (*Triticum aestivum*), since Put decreased under saline stress whereas a significant increase in Spd and Spm occurred (Capell et al., 2004; El-Shintinawy, 2000; Maiale et al., 2004). However, the results found here are most consistent with a salt stress study in the monocot grass species rice (*Oryza sativa*), in which salt sensitivity of a rice cultivar was associated with accumulation of Put and low level of Spd and Spm (Krishnamurthy and Bhagwat, 1989). Creeping bentgrass 'PsgSLTZ' overall exhibited less production of Put in response to salt stress, which could be associated with a greater salt tolerance. However, the

cultivar differences in salt tolerance were not fully clear. Thus, it seems that these two creeping bentgrass cultivars are exhibiting a trend in PA content changes that are associated with salt sensitivity. Therefore, supplementation of PA biochemical pathways via exogenous application of PAs or by genetic modification may be beneficial for creeping bentgrass survival of salt stress.

In conclusion, both types of creeping bentgrass evaluated exhibited a significant decline in plant health due to salt treatment. PA content was also affected by salt stress treatment. Overall, Put increased due to salt stress whereas Spd was generally unaffected and Spm had a transient increase in content due to salt stress. Since creeping bentgrass exhibits PA regulation that may be associated with stress sensitivity, we are currently evaluating whether the enhancement of PA regulation in creeping bentgrass, such as through exogenous application or molecular techniques may be a viable method of improving creeping bentgrass tolerance to salt. In our recent work, we have found that exogenous application of PAs did improve creeping bentgrass responses to drought stress (Shukla et al. 2015). More work is needed to elucidate the physiological effects of PAs in relation to abiotic stresses in turfgrass species.

APPENDIX

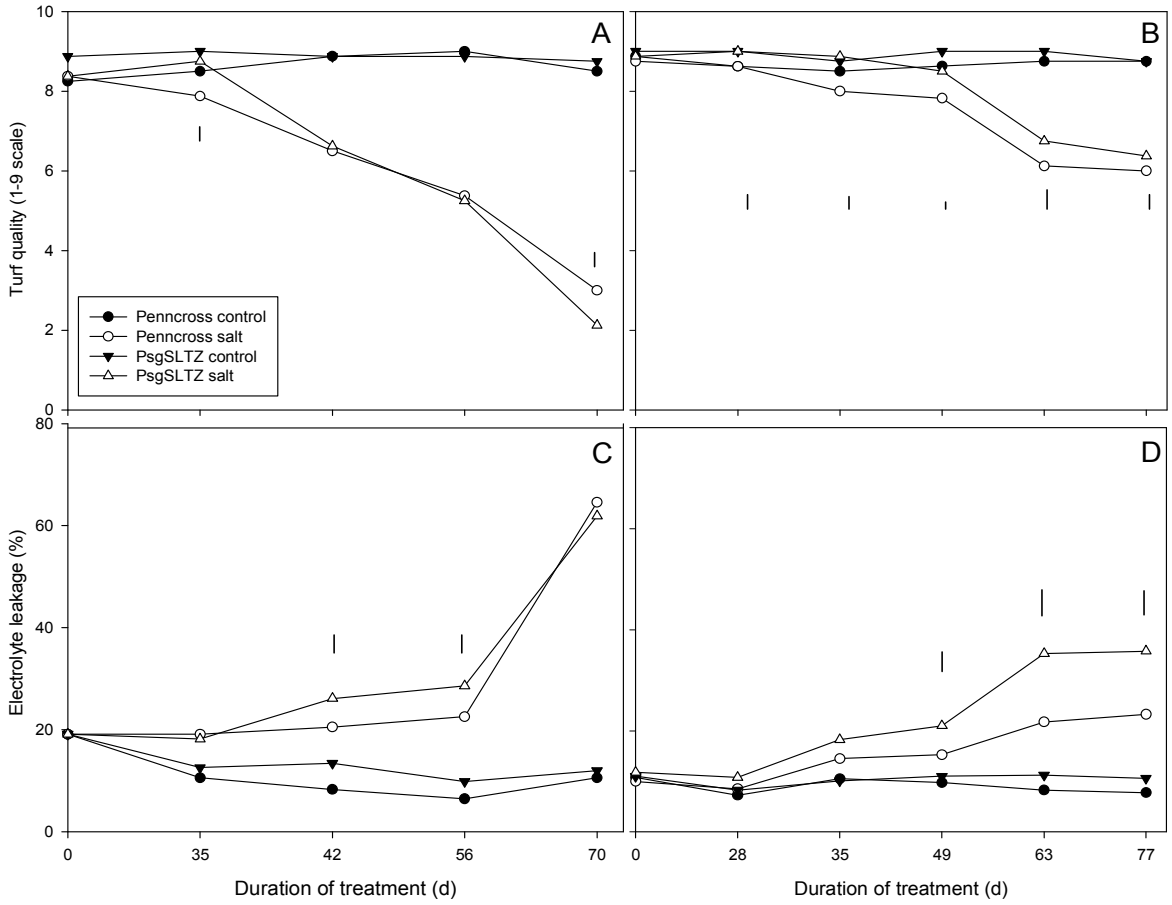

Figure 1: Turf quality rating of creeping bentgrass 'PsgSLTZ' and 'Penncross' exposed to salt stress treatment in (A) expt. 1which was started on Dec 21 2013 and (B) expt. 2 which was started on May 6 2014. Percent electrolyte leakage of leaves in (C) expt. 1 and and (D) expt. 2. Treatment means were separated using Fisher's LSD (n = 4) ($P \leq 0.05$), which is represented by the vertical bar. Rating was from 1= poor to 9=excellent based on turf color, uniformity, and density.

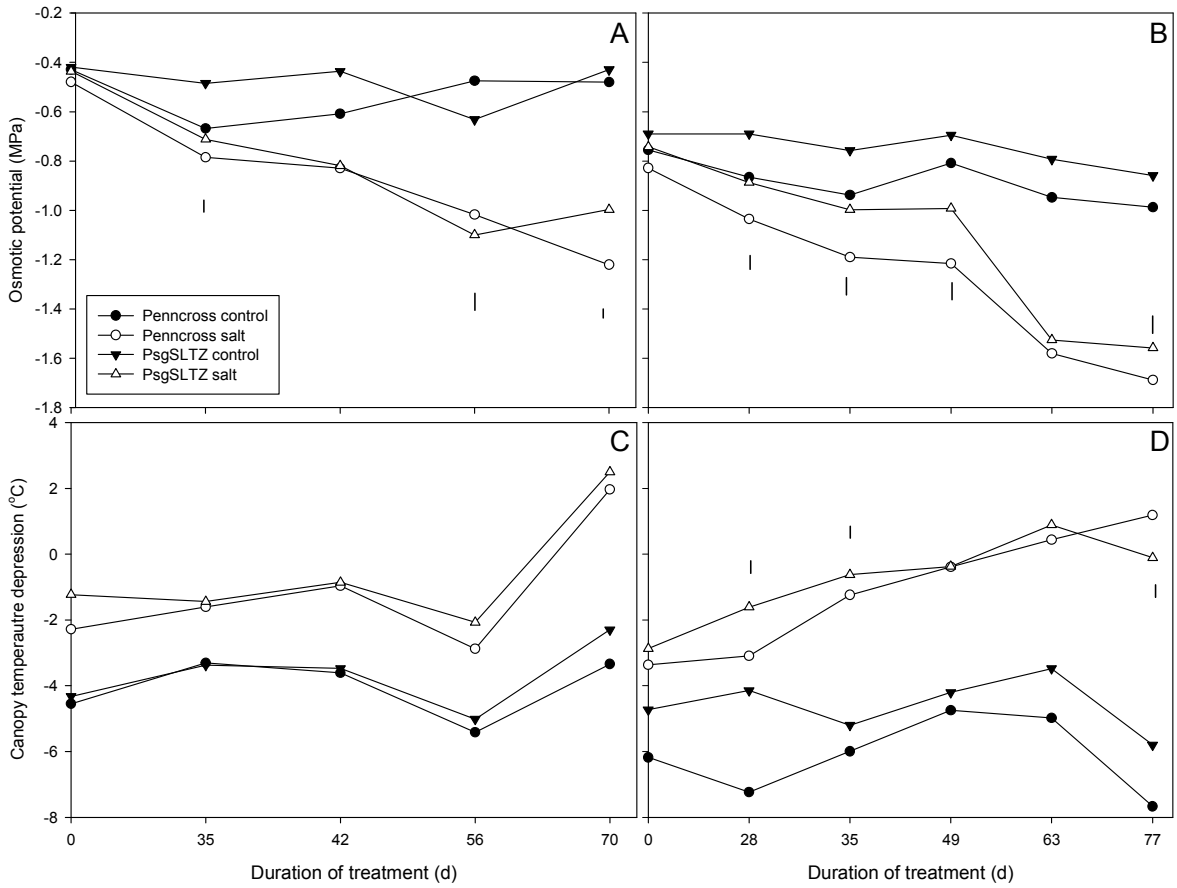

Figure 2: Leaf osmotic potential of creeping bentgrass 'PsgSLTZ' and 'Penncross' exposed to salt stress treatment in (A) expt. 1, which was started on Dec 21 2013, and (B) expt. 2, which was started on May 6 2014. Canopy temperature depression presented in (C) expt. 1 and (D) expt. 2. Treatment means were separated using Fisher's LSD (n = 4) ($P \leq 0.05$), which is represented by the vertical bar.

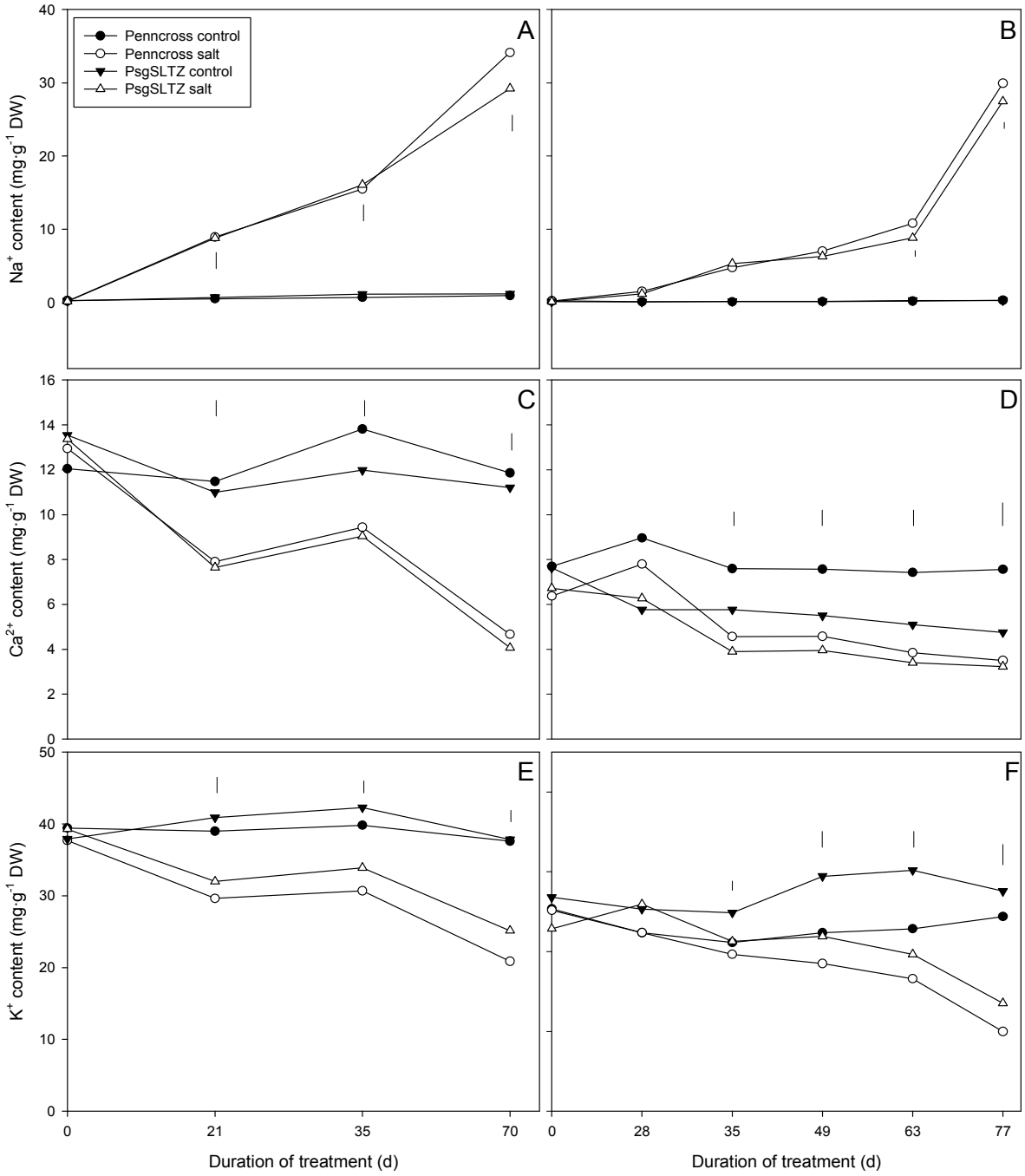

Figure 3: Leaf mineral content of creeping bentgrass 'PsgSLTZ and 'Penncross' under salt stress. Leaf content of (A) Na^+ (C) Ca^{2+}, and (E) K^+ in 'PsgSLTZ' and 'Penncross' from expt. 1 (B) Na^+ (D) Ca^{2+}, and (F) K^+ for expt. 2. Treatment means were separated using Fisher's LSD (n = 4) ($P \leq 0.05$), which is represented by the vertical bar. Day 0 for expts. 1 and 2 started on Dec 21 2013 and May 6 2014, respectively.

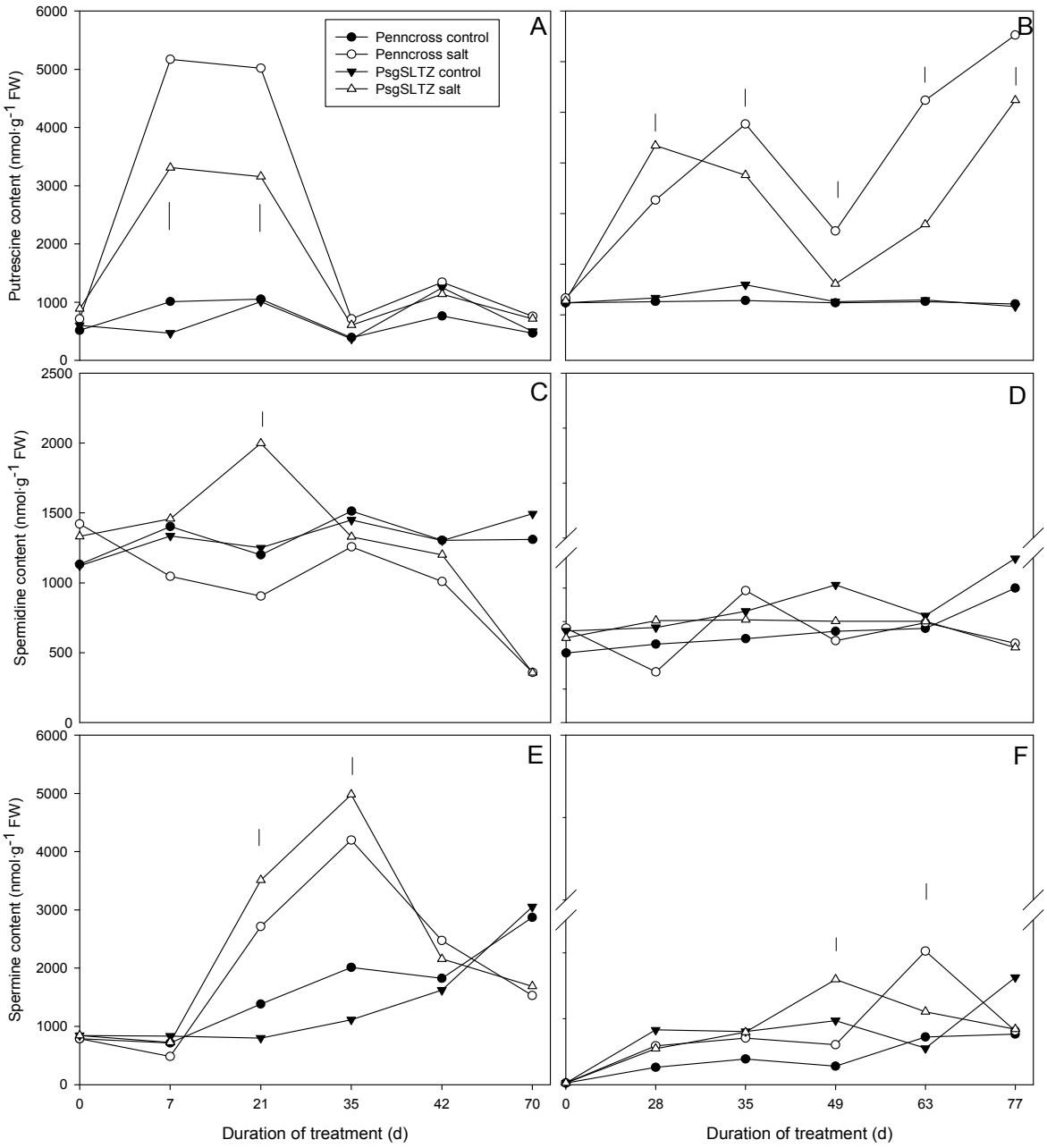

Figure 4: Content of free polyamines in leaves of creeping bentgrass 'PsgSLTZ' and 'Penncross' under salt stress. Putrescine content in (A) expt. 1 and (B) expt. 2. Spermidine content in (C) expt. 1 and (D) expt. 2. Spermine content in (E) expt. 1 and (F) expt. 2. Treatment means were separated using Fisher's LSD (n = 4) ($P \leq 0.05$), which is represented by the vertical bar. Day 0 for expts.1 and 2 started on 21 Dec. 2013 and 6 May 2014, respectively.

Table 1: Analysis of variance (ANOVA) in type III error tests of fixed effects on various traits analyzed under different days after salt treatment for expt. 1 (0, 35, 42, 56, and 70) and expt. 2 (0, 28, 35, 49, 63, and 77).

		Experiment 1 (days)					Experiment 2 (days)					
Traits	**Effect**	**0**	**35**	**42**	**56**	**70**	**0**	**28**	**35**	**49**	**63**	**77**
	Salt	ns	***	***	***	***	ns	ns	ns	***	***	***
TQ	**Cul**	ns	***	ns	ns	ns	ns	*	***	***	ns	ns
	Salt*Cul	ns	ns	ns	ns	*	ns	ns	*	ns	ns	ns
	Salt	ns	ns	ns	***	***	ns	*	ns	***	***	***
EL	**Cul**	ns	ns	***	ns	ns	ns	*	*	***	***	***
	Salt*Cul	ns	ns	***	ns	ns	ns	ns	ns	ns	**	*
	Salt	ns	***	ns	***	***	ns	ns	***	***	***	***
RWC	**Cul**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Salt*Cul	ns	ns	ns	ns	ns	ns	ns	ns	***	ns	ns
	Salt	ns	*	***	***	ns	ns	***	***	***	***	***
Ψ	**Cul**	ns	ns	ns	**	***	ns	***	***	***	*	***
	Salt*Cul	ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns
	Salt	ns	***	***	***	***	ns	***	***	***	***	***
CTD	**Cul**	ns	ns	ns	ns	ns	ns	***	*	ns	*	ns
	Salt*Cul	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Salt	ns	ns	ns	**	ns	ns	ns	ns	*	ns	ns
CHL	**Cul**	ns	ns	ns	ns	ns	ns	ns	***	ns	***	ns
	Salt*Cul	ns	ns	ns	*	*	ns	ns	ns	ns	ns	ns

TQ indicates overall canopy turf quality rating based on the scale of 1 to 9; EL indicates percentage of leaf electrolyte leakage; RWC indicates leaf relative water content; Ψ indicates leaf osmotic potential; CTD indicates the canopy temperature depression; CHL indicates the leaf chlorophyll content; Cul indicates the effect of cultivar; Salt indicates the effect of salt treatment; Salt*Cul indicates the effect of interaction between salt and cultivar. NS indicates a non-significant difference was detected; *, **, *** represents significant effect at the level of 0.05, 0.01, and 0.001, respectively.

Table 2: Analysis of variance (ANOVA) in type III error tests of fixed effects on various traits analyzed under different days after salt treatment for expt. 1 (0, 21, 35, and 70) and expt. 2 (0, 28, 35, 49, 63, and 77).

		Experiment 1(days)				Experiment 2 (days)					
Traits	**Effect**	**0**	**21**	**35**	**70**	**0**	**28**	**35**	**49**	**63**	**77**
	Salt	ns	***	***	***	ns	***	***	***	***	***
Na	**Cul**	ns	ns	ns	***	ns	ns	***	***	*	ns
	Salt*Cul	ns	ns	ns	***	ns	ns	***	***	*	ns
	Salt	ns	***	***	***	ns	ns	***	***	***	***
K	**Cul**	ns	ns	***	ns	ns	***	***	***	***	***
	Salt*Cul	ns	ns	ns	ns	ns	*	ns	***	***	ns
	Salt	ns	***	***	***	ns	***	***	***	***	*
Ca	**Cul**	ns	ns	*	ns	ns	***	***	***	***	***
	Salt*Cul	ns	ns	ns	ns	ns	***	***	***	***	ns

Na indicates leaf sodium content; K indicates leaf potassium content ; Ca indicates leaf calcium content; Cul indicates the effect of cultivar; Salt indicates the effect of salt treatment; Salt*Cul indicates the effect of interaction between salt and cultivar. NS indicates a non-significant difference was detected; *, **, *** represents significant effect at the level of 0.05, 0.01, and 0.001, respectively.

Table 3: Analysis of variance (ANOVA) in type III error tests of fixed effects on various traits analyzed under different days after salt treatment for expt. 1 (0, 7, 21, 35, 42, and 70) and expt. 2 (0, 28, 35, 49, 63, and 77).

		Experiment 1(days)						Experiment 2(days)					
Traits	**Effect**	**0**	**7**	**21**	**35**	**42**	**70**	**0**	**28**	**35**	**49**	**63**	**77**
	Salt	ns	ns	ns	*	ns	ns	ns	ns	***	ns	***	***
Put	**Cul**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns
	Salt*Cul	ns	ns	ns	ns	ns	ns	ns	***	ns	ns	**	ns
	Salt	ns	ns	ns	ns	*	***	ns	ns	ns	ns	ns	***
Spd	**Cul**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Salt*Cul	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Salt	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
Spm	**Cul**	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*
	Salt*Cul	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	*

Put indicates leaf putrescine content; Spd indicates leaf spermidine content; Spm indicates leaf spermine content; Cul indicates the effect of cultivar; Salt indicates the effect of salt treatment; Salt*Cul indicates the effect of interaction between salt and cultivar. NS indicates a non-significant difference was detected; *, **, *** represents significant effect at the level of 0.05, 0.01, and 0.001, respectively.

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CHAPTER 3

CREEPING BENTGRASS RESPONSES TO DROUGHT STRESS AND POLYAMINE APPLICATION

Abstract

Polyamines (PAs) such as spermidine (Spd), spermine (Spm) and putrescine are involved in various biological functions including abiotic stress response. Whether PAs play an important role in cool-season turfgrass tolerance of drought stress is not well investigated. We have conducted a series of growth chamber (GC) studies including one hydroponic and two soil based GC studies with creeping bentgrass 'Penncross' (*Agrostis stolonifera*) and 'Penn-G2' to determine whether exogenous application of PAs may affect plant growth and stress tolerance. Application of relatively low concentrations of Spd (500 or 750 $\mu M \cdot L^{-1}$) or Spm (500 $\mu M \cdot L^{-1}$) promoted tillering rates under optimal growth conditions in hydroponics. The same levels of PA treatments moderated the damages associated with drought stress in the soil based GC studies. The most notable differences in drought response associated with PA treatment were increased membrane health. This was observed as greater photochemical efficiency, higher quantum yield, less electrolyte leakage, and less lipid peroxidation (malondialdehyde content) in PA treated plants compared to control plants. The relatively low level of exogenous PAs used in this study did not have a major effect on plant water relations under drought stress. Canopy temperatures and soil moisture content were not affected by any PA treatment; however, on some days during early drought stress relative water content was significantly higher in PA treated plants compared to controls. PA could play a major role in protecting photosynthetic and cellular membranes during drought stress of creeping bentgrass.

Key words: *Agrostis stolonifera*, water stress, turfgrass, tillering, spermidine, spermine, photochemical efficiency, quantum yield, lipid peroxidation

Introduction

Plant cells contain long chain amine compounds called PAs, of which the most common are Spd, Spm, and putrescine. These compounds are considered phytohormone-like since they play various regulatory roles in plant cells. They are involved in controlling cell development, in stress responses, and numerous other functions (Gill and Tuteja, 2010). Natural increases or decreases in PA content occur in plant cells in response to stress. For example, a halophyte grass called vetiver grass (*Chrysopogon zizanioides*) was shown to accumulate levels of Spd and Spm under drought stress whereas putrescine levels decreased (Zhou and Bingjun, 2009). Whether PAs accumulate due to stress damage or in order to protect plant cells from stress is still not well understood in most species (Capell et al., 2004). Since salt tolerant vetiver grass utilizes PA pathways for stress tolerance, it may be desirable to understand whether PAs have a similar protective role in high value, cool-season turfgrass species such as creeping bentgrass. Additionally, PA fluxes are differential among species during stress incidence. Arabidopsis (*Arabidopsis thaliana*) plants compared to resurrection (*Craterostigma plantagineum*) plants tended to shift in opposite directions along the arginine decarboxylase pathway of PA biosynthesis during stress (Alcazar et al., 2011). These species differences in PA pathway fluxes during a stress incident could be related to differences in each species' primary mechanism utilized to cope with drought stress; whether there is a tendency towards escape, avoidance, or tolerance mechanisms. Thus, investigation of responses to PA in multiple species is necessary and could indicate preferential drought survival mechanisms among species.

A primary factor related to a plant's drought coping mechanism is related to tendency for ethylene production and leaf senescence (Chaves et al., 2003). PAs and ethylene are closely linked in plant metabolic pathways, since they all use S-adenosyl methionine (SAM) as precursors for biosynthesis. PAs and ethylene act antagonistically since they are competing for SAM substrates. Research has shown that PAs do promote opposite cellular processes than ethylene by promoting growth and delaying senescence (Bitrián et al., 2012; Torrigiani et al., 2012). In creeping bentgrass, delaying leaf senescence with a transgene for cytokine biosynthesis was found to improve drought stress tolerance (Merewitz et al., 2010, 2011). Here, we are investigating whether PAs may have similar protective effects on improving abiotic stress tolerance in creeping bentgrass.

Genetically modified enhancement of the biosynthesis of some forms of PAs has been shown to be effective in promoting plant tolerance to abiotic stresses. For instance, increases in Spd and Spm levels were shown to confer tolerance to drought in transgenic rice [*Oryza sativa* (Capell et al., 2004)], high temperature stress in tomato [*Solanum lycopersicum* (Cheng et al., 2009)], as well as salt stress in corn [*Zea mays* (Jiménez-Bremont et al., 2007)]. Overexpression of a Spd synthase gene in Arabidopsis plants improved plant tolerance to many abiotic stresses including drought stress (Kasukabe et al., 2004); however, endogenous content of PAs are sensitive to plant environmental stimuli and turfgrass species are culturally managed significantly differently than in other crop species (most notably mowing; Beard, 2006). Thus, an investigation of PAs specifically in cool-season turfgrass species is needed to determine whether these compounds may be important to the turfgrass industry.

Exogenous application of PAs has been shown to be an effective strategy to increase plant tolerance of various stresses and regulate plant growth. For instance, salt stress damage

in rice and water loss in mandarin orange (*Citrus reticulata*) was mitigated by exogenous application of PAs (Shi and Chan, 2014). There also could be potential changes in growth regulation since PAs are involved in cell division, differentiation, and DNA replication processes for plant cell growth (Kusano et al., 2008). Recently, there is evidence that there are rhizobacteria that may exude PAs to alter plant growth responses (Xie et al., 2014). It is not known whether PAs play a role in regulating turfgrass species growth of shoots or roots. Understanding this could have major implications for plant growth regulator (PGR) products in the turfgrass industry. Additionally, whether PAs have any PGR effect on turfgrass species is an essential step before evaluating responses of a turfgrass to PA and stress, since differential tillering could be mistaken for increased drought tolerance. PA application and abiotic stress responses were recently evaluated in a warm-season grass bermudagrass (*Cynodon dactylon*), which exhibited improvements in drought and salt tolerance in response to relatively high levels of PA application (Shi et al., 2013). Growth responses and photochemical attributes were not evaluated in their study and they used a relatively high rate of PA application (5 mM). Specific investigation of whether cool-season turfgrass species such as creeping bentgrass will be affected by exogenous PA application is desirable. Cool-season grasses differ significantly from warm-season grasses in growth habit, biochemical processes, and tolerance to abiotic stress. Additionally, whether a relatively low concentration of PA is effective in mitigating stress damages is not known and would be most feasible in practical applications.

Drought stress is one of the most commonly encountered abiotic stresses in the turfgrass industry. Drought stress readily occurs even on irrigated areas in times of limited rainfall, due to water use restrictions, on areas with poor irrigation instrumentation, and due to

the high demand for firm dry conditions for adequate playability of turf areas such as golf courses. The objectives of this series of growth chamber studies were to investigate whether foliar applications of Spm (500 $\mu M \cdot L^{-1}$) and Spd (500 or 750 $\mu M \cdot L^{-1}$) have an effect on creeping bentgrass growth under optimal conditions and under drought stress. Our objectives were to determine whether PAs alter growth characteristics such as leaf and root biomass, tillering rate, and stress protective properties such as improving cellular water status and reducing drought damage to cellular membranes.

Material and methods

Plant material and measurements – hydroponic experiment

Plugs of 'Penncross' creeping bentgrass were taken from the Hancock Turfgrass Research Center in East Lansing, MI and were separated into single tillers in order to obtain clonal material for a hydroponic experiment. The single tillers were established in hydroponic system in early Aug. 2013 and were allowed to grow without any experimental treatments until adequate root and shoot development occurred. The PA treatment began on 29 Aug. 2013 and the experiment terminated on 3 Oct. 2013. The hydroponics study was performed in order to enable a determination of growth regulator effects of PAs on shoot and root attributes of creeping bentgrass plants without having to majorly disturb or damage plants such as would occur in a soil based system. The hydroponic growth media was contained within opaque containers (38 x 28 x 16 cm) within a large walk-in controlled environment growth chamber. The growth chamber conditions are the same as described below for the soil-based GC studies. The hydroponic growth media was made via dilution of stock solutions (1000X) of the following nutrient solutions: 71.361 $g \cdot L^{-1}$ $(NH_4)_2 SO_4$, 27.3 $g \cdot L^{-1}$ KNO_3, 127.521 $g \cdot L^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$, 68.045 $g \cdot L^{-1}$ KH_2PO_4, 43.568 $g \cdot L^{-1}$ K_2SO_4, 199.65 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$,

14.684 g·L^{-1} Fe(EDTA)Na, 1.43 g·L^{-1} H_3BO_3, 0.91 g·L^{-1} $MnCl_2 \cdot 4H_2O$, 0.11 g·L^{-1} $ZnSO_4 \cdot H_2O$, 0.04 g·L^{-1} $CuSO_4$, 0.01 g·L^{-1} $(NH_4)MO_7\ O_{24} \cdot 4H_2O$. Single tillers of plants were suspended within holes (1 cm) on an expandable sponge in a polystyrene foam board. The board was cut to fit the containers exactly to prevent light from reaching the root system. The hydroponic solution was aerated with a tube inserted into the solution through the foam board connected to a pump (115V, 60Hz; Tetra®, Blacksburg, VA). Solution pH was monitored and adjusted as needed every other day (to maintain a pH of approximately 6) and the solution was changed on a weekly basis. Plants were not clipped to allow for leaf and root measurements, as to not alter natural tillering rate due to clipping.

Polyamines treatments for the hydroponic growth chamber study included inclusion of the following concentrations of PAs in the hydroponic growth solution 1) no PAs control, 2) 100 μM·L^{-1} Spd, or 3) 500 μM·L^{-1} Spd. Each plant was individually treated with the respective PA solution or control (no PA) by dipping the full root system of each plant into the solution for 5 min, patted dry on paper towel, and then rinsed with water for 2 min. Due to space limitations and laborious nature of this analysis we did not treat with all PAs discussed in this paper. Spd was chosen as it is one of the most central PAs within the PA biosynthesis pathways and was found in previous studies to have an effect on plant growth (Ahmed et al., 2012). Leaf growth rate measurements were performed manually by counting leaf number and tiller number. Root growth measurements were taken on intact plants by placing the root system in a clear tray of water placed on a scanner. Roots were separated and scanned using a flatbed scanner. Root quantification was performed using Digimizer software (version 4.3; MedCalc software; Ostend, Belgium) on scanned images of intact plants to determine root number and root length. Root: shoot ratio and total biomass were recorded and calculated by

weighing plants on a balance at the end of the study. Chlorophyll content was determined with a handheld chlorophyll meter (SPAD 502 Plus; Spectrum Technologies, Aurora, IL).

Plant material and growth conditions – soil based experiments

Mature sod pieces of creeping bentgrass 'Penncross' and 'Penn-G2' were collected from the Hancock Turfgrass Research Center in East Lansing, MI and evaluated in two independent growth chamber experiments. The two growth chamber studies (GC1, GC2) were performed in soil in pots. GC1 was conducted on 'Penncross' and GC2 was conducted on 'Penn-G2.' GC1 study started on 25 Oct. 2013 and terminated on 6 Nov. 2013. GC2 studies on 'Penn-G2' were initiated on 7 Feb. 2014 and ended on 19 Feb. 2014. These cultivars were selected based on availability and since 'Penn-G2' is touted to be a more drought tolerant cultivar compared to 'Penncross.' For both of the soil based chamber studies, the plant material and growth chamber conditions were equivalently handled and maintained. Sod pieces were potted in polyvinyl chloride tubes (40 cm height x 10.16 cm diameter) in 1:1 fine sand: soil mix. The soil was a sandy loam (Typic Hapludult) with a pH of 7.7 based on soil particle size/sand classification test results. Plants were allowed to become established and were maintained in a greenhouse for approximately 1 month. The plants were then transferred to an environmentally controlled growth chambers where treatments were initiated after a 2-week acclimation period. The chamber conditions were set to maintain a 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light level with 24/18 ^{o}C day/night temperature and a 60% relative humidity. Plants were fertilized twice per week with half strength Hoagland solution (Hoagland and Arnon, 1950). Plants were trimmed weekly to attain a dense, even sized canopy. The canopy was trimmed twice per week to maintain a height of 5 cm.

Drought and polyamine treatments – GC1, GC2

Water treatments included either a well-watered control or drought stress imposed by complete withholding of water in both growth chamber studies. Each polyamine treatment under either drought stress or watered conditions included 4 biological replicates. Foliar treatments of PAs in GC1 and GC2 included 1) water control, 2) 500 $\mu M \cdot L^{-1}$ Spd (Spd500), 3) 750 $\mu M \cdot L^{-1}$ Spd (Spd750), and 4) 500 $\mu M \cdot L^{-1}$ Spm (Spm500). These concentrations were selected based on reports in other crop species such as Ahmed et al. (2012). Exogenous application of freshly made PA or control solutions were applied with an atomizer sprayer until run-off from the leaves occurred (40 mL per plant) and all solutions contained 0.125% Tween-20 (Sigma-Aldrich, St. Louis, MO). PA solutions had a pH of approximately 8. All PA solutions were made just prior to application to the plants and for the soil based GC studies they were sprayed once just prior to subjecting the plants to drought stress (0 d). Plants of each cultivar and PA treatment were subjected to drought stress one day following PA treatment. Watering treatments included 1) well-watered control plants and 2) drought stressed plants (water completely withheld). The drought period lasted for a total of 12 d for GC1 and GC2. The experiments were allowed to go on until severe damage to plants was observed and the experiments were terminated for destructive harvesting. Therefore, a total of 32 plants were used for each GC1 and GC2.

Soil moisture and physiological measurements - GC1, GC2

The volumetric water content of the soil (SWC) for GC1 and GC2 was measured by a time domain reflectometry (TDR) using a 9 cm probe connected to a data logger (TDR 100; Spectrum Technologies). The TDR probe was inserted into the upper portion of the soil during monitoring.

To determine relative water content of the leaves (RWC) 5-10 fully expanded mature leaves were collected from each plant and weighed to record fresh weight (FW). To determine turgid weight (TW) of the same samples, leaves were wrapped in tissue paper and soaked in de-ionized water at 4 ºC for approximately 24 h to attain full hydration. Then samples were blotted dry with paper towels and weighed immediately to determine TW. Leaf samples were stored at 65 ºC for 2 weeks to dry to determine dry weight (DW). Leaf RWC was calculated using equation: (FW-TW)/ (TW- DW) X 100 (Barrs and Weatherley, 1962).

Turf quality (TQ) was visually rated based on color, density and overall plant health on a scale of 1-9, with 1 being completely brown and dehydrated, 5 as moderately drought affected turf and 9 as healthy, green, dense canopy (Turgeon, 2008).

Canopy temperature depression (CTD; Rashid et al., 1999) was calculated as the difference between the air and turf canopy temperature, which were monitored using a ambient temperature thermometer positioned in the growth chamber at canopy level and an infrared thermometer was used to measure canopy temperature (model 2956; Spectrum Technologies).

Leaf electrolyte leakage (EL) was measured by taking approximately 200 mg of leaf samples from each plant. The tissue was briefly rinsed in deionized water and then put into 15-mL tubes carrying 7 mL of deionized water. The samples were then placed on a shaker at room temperature overnight. The initial level of conductance (C_i) was measured. Then the samples were boiled at 99ºC for 4h to kill the leaf tissue and brought to room temperature with constant shaking for another 24 h. The conductivity of the water containing the dead tissues was measured as the maximum conductance (C_{max}). The percentage EL was calculated as C_i/C_{max} x 100 (Blum and Ebercon, 1981).

Membrane health attributes were estimated by measuring the following parameters. Leaf photochemical efficiency (F_v/F_m) and quantum yield of photochemical energy conversion in PSII (YII) were determined with a fluorometer system (OSp5; Opti-Sciences, Hudson, NH) on individual, fully expanded leaves by taking three subsamples per plant. Leaf malondialdehyde content (MDA) to estimate lipid peroxidation level was determined with the method obtained from Dhindsa et al. (1981) with modifications. A 1.0 mL of enzyme solution was added to 2 mL of reaction solution containing 20% (v/v) trichloroacetic acid and 0.5% (v/v) thiobarbituric acid. The solution was heated in a water bath at 95 ^{o}C for 30 min, quickly cooled on ice, and centrifuged at 10,000 g_n for 30 min. A spectrophotometer (Genesys 20; Thermo Scientific, Waltham, MA) was used to measure absorbance of the solution at 532 and 600nm. MDA content was calculated by 600 nm subtracted from absorbance at 532 nm and then using this adjusted absorbance and extinction coefficient of 155 mM $^{-1}$cm^{-1} (Heath and Packer, 1968).

Experimental design and statistical analysis

All GC experiments were a completely randomized block design including four biological replicates for GC1, GC2 or 6 replicates for hydroponic growth chamber study for each treatment. Effects of the chemical and drought treatment were analyzed over time by analysis of variance (ANOVA) based on the general linear model procedure of SAS (version 9.1; SAS Institute, Cary, NC). Fisher's protected least significant difference (LSD) test at $P \leq 0.05$ to detect differences between treatment means.

Results

Hydroponic growth study

The only growth regulator effect that occurred in response to PA application was a change in leaf number and tillering rate. A significantly greater number of total leaves were observed due to the presence of 500 $\mu M \cdot L^{-1}$ Spd in the culture media at 35 d of growth (Figure 5A). The rate of tillering was stimulated by Spd at 500 $\mu M \cdot L^{-1}$ treatment at 28 and 35 d of growth (Figure 5B). Tillering rate was not significantly affected by all PA treatments (only Spd 500 $\mu M \cdot L^{-1}$) or on all dates. We found no significant differences in root biomass, root to shoot ratio, or leaf length (data not shown).

Water relations

SWC declined markedly in response to withholding water from drought treated plants in both GC studies. SWC declined rapidly to approximately 5% in each study (Figure 6). There were no significant differences among chemical treatments in SWC either among well-watered plants or among drought stressed plants in either of the GC studies (Figure 6). Drought stress caused a significant decrease in CTD for both 'Penncross' and 'Penn-G2'. Higher canopy temperatures were detected in drought stressed plants compared to well-watered plants; however, no significant differences were detected for CTD among chemical treatments (data not shown). RWC of plants decreased dramatically due to drought stress of both 'Penncross' and 'Penn-G2'. Significant differences among PA treatments within plants under drought stress occurred on 3, 5, and 7 d of drought stress during GC1 study of 'Penncross' (Figure 6C) and on 5 and 8 d during GC2 study of 'Penn-G2' (Figure 6D). For instance, plants treated with Spm500 had a significantly higher RWC compared to the control plants on 5 and 7 d of drought stress of 'Penncross'. In 'Penn-G2', on 5 d drought stress all PA treated plants had a

significantly greater RWC than control plants. By 8 d of drought stress this difference was less apparent, since only drought stressed Spd750 and Spm500 plants were significantly higher in RWC than control plants. Overall, 'Penn-G2' had a more marked response to PA treatment compared to 'Penncross' in terms of maintenance of RWC of leaves.

Turf quality, membrane, and photochemical health

Well-watered plants maintained relatively consistent levels of TQ, EL, F_v/F_m and YII in both GC1 and GC2. No significant differences in any of these parameters occurred due to treatment with PA. For drought stress treated plants, the TQ, F_v/F_m and YII all declined markedly whereas EL increased (Figures 7 and 8). Drought stressed plants treated with PA had significantly higher TQ in both GC1 and GC2 for the duration of the drought period (Figure 7A and B). The TQ of 'Penncross' in GC1 was improved the most by treatment with Spm500 under drought stress. In GC2 with 'Penn-G2', treatment with Spd750 and Spm500 exhibited the greatest maintenance of TQ. Spd500 also improved TQ during drought stress of 'Penn-G2' but to a more moderate extent.

EL was increased to a greater extent in drought stressed control plants compared to those plants treated with PAs in both GC studies (Figure 7C and D). For GC1 with 'Penncross', EL was not significantly affected on all dates of drought stress, but there were significant reductions of EL due to treatment with Spm500 such as on 5 and 9 d of drought. A more dramatic separation of EL was observed in drought stressed 'Penn-G2' plants in GC2. In this study, Spd500 was the most effective PA treatment since it consistently reduced EL of leaves on 5, 8, and 12 d of drought stress.

F_v/F_m and YII were greatly reduced by drought stress in both cultivars of creeping bentgrass. Treatment with all tested PAs delayed the reduction in photochemical health (F_v/F_m

and YII). For instance at 7 d of drought in 'Penncross' and 8 d of drought in 'Penn-G2', PA treated plants had two to three times higher F_v/F_m compared to control plants (Figure 8A and B). Similar results were observed for YII throughout the duration of drought treatment (Figure 8C and D).

Lipid peroxidation (MDA content) was unchanged in well-watered plants whereas MDA increased significantly in drought treated plants in both GC studies (Figure 9). Overall, the MDA content increased more dramatically in control plants compared to PA treated plants; however, this was not significant on all dates for all treatments. In GC1 for 'Penncross' (Figure 9A), Spd750 and Spm500 plants had less MDA accumulation compared to control drought treated plants on 5 and 8 d of drought stress. Spd500 only showed significantly less MDA content after 8 d of drought. In GC2 for 'Penn-G2' (Figure 9B), no significant difference in MDA content was detected until 8 d of drought treatment. On 8 d, all PA treated drought stressed plants had significantly lower accumulation of MDA compared to control drought stressed plants.

Discussion

To understand whether PA treatment has a growth regulator type effect on creeping bentgrass plants, we have conducted a hydroponic study to determine whether changes in leaf biomass, root biomass, and tillering rate due to PA treatment under optimal conditions. Whether PA application played a major role in growth regulation is still not fully clear from the results of this study. We found that tillering rate was greater in Spd at the 500 $\mu M \cdot L^{-1}$ level at 28 and 35 d of growth in the hydroponic system; however, no other growth parameters were significantly affected in leaves or roots due to PA treatment. Regardless of this, we have taken into account this potential change in tillering when interpreting the responses to drought stress.

We have done this by eliminating the density component of TQ ratings that is typically done when rating turfgrasses (Turgeon, 2008). Thus, our TQ ratings are based primarily on green color, uniformity, and degree of wilting. Specific effects of PA treatment on tillering rate of grass or other plant species is not well-documented. It is generally known that PAs supplementation may stimulate plant growth and development through regulation of cell division and elongation (Kusano et al., 2008). Wheat (*Triticum aestivum*) plants exhibited a significant increase in growth characteristics in response to 50-100 $mg \cdot L^{-1}$ of putrescine and Spd treatment (Ahmed et al., 2012). More detailed studies related to creeping bentgrass tillering and growth rate related to different concentrations of PA content may be desirable.

Due to drought stress, creeping bentgrass plants exhibited significant stress symptoms and decline in health in both growth chamber studies that were conducted. As the SWC declined during the drought treatment, rapid cellular water loss occurred, which was apparent in the rapid reductions in RWC of creeping bentgrass leaves. Based on the parameters that were measured, one of the major consequences of drought was damage to cellular membrane structures, including the cellular membrane and photosynthetic membrane constituents. This is consistent with previous studies on drought stress of creeping bentgrass (Chai et al., 2010; Merewitz et al., 2011).

Based on the results of both GC studies, the PA treatments did not have a major effect on all water relations of creeping bentgrass under drought stress. The SWC declined at a similar rate for all plants in the studies and no significant differences were detected for CTD among chemical treatments. RWC of the leaves was found to be less in control plants compared to those treated with PA during the earlier stages of drought stress. This difference was more pronounced in 'Penn-G2' compared to in the study of 'Penncross'. Treatments with

either Spm or Spd at the 500μM·L^{-1} level delayed leaf water loss during drought stress. These differences were not detectable by 10 (GC1) or 12 (GC2) d of drought treatment. The lack of effect of PAs on all water relations in this study could be related to the low rate of PA treatment utilized. Future work will include testing greater rates of PA to determine if there is a more marked effect on water relations during drought stress of turfgrasses.

The most readily observable response to PA treatments during drought stress was a significant improvement in the photochemical health of creeping bentgrass. In both growth chamber studies, we have observed a marked delay in the reduction of F_v/F_m and YII caused by drought stress in PA treated plants. This finding is consistent with previous literature that has noted that one major mode of PA action is on photochemical pathways within chloroplasts (Ioannidis and Kotzabasis, 2007). These researchers revealed that the concentration of applied Spd and Spm can affect that action of these compounds in chloroplast membrane photosystems. For instance, Spd and Spm enhance non-photochemical quenching at lower rates whereas they uncouple photo phosphorylation at higher concentrations. Duan et al (2008) also found PAs to play a major role in preventing membrane damage in salt-stressed cucumber plants (*Cucumis sativus*). A promotion of F_v/F_m health has been determined to be a major factor associated with drought tolerance of creeping bentgrass in many previous reports (Chai et al., 2010; McCann and Huang, 2008). We think that the marked increase in photochemical health and a moderate maintenance of RWC in some treatments could have been the major component that improved TQ ratings for PA drought stressed plants compared to control drought stressed plants. A delay in senescence could be related to maintenance of TQ but more work is needed to determine PA association with ethylene and leaf senescence in creeping bentgrass. In peach (*Prunus persica*) fruits,

application of Spd was shown to delay the ripening process controlled by ethylene (Torrigiani et al., 2012). Consistent with the results of PA providing protection to the cellular membranes, we found that a lower amount of lipid peroxidation occurred in PA treated plants under drought stress. Less MDA has also been associated with less leaf senescence (Dhindsa et al., 1981); however, whether MDA content was related to senescence cannot be directly concluded from the measurements done in this study. Similar results of PA application reducing MDA content was found associated with drought and salt stress of bermudagrass (Shi et al., 2013). Analysis of antioxidant enzyme activity and other membrane protective mechanisms in response to PA treatment may be of use to further discuss this finding in creeping bentgrass.

Low level of PA treatment mitigated some drought damage and moderately improved cellular water retention and photochemical health of creeping bentgrass. Since PAs seem to play a role in drought tolerance of creeping bentgrass we are currently investigating how PA content changes due to stress and how phytohormones may relate to shifts in PA content. Additionally, studies of how PAs are altered by common management practices of turfgrass culture will be conducted in the future.

APPENDIX

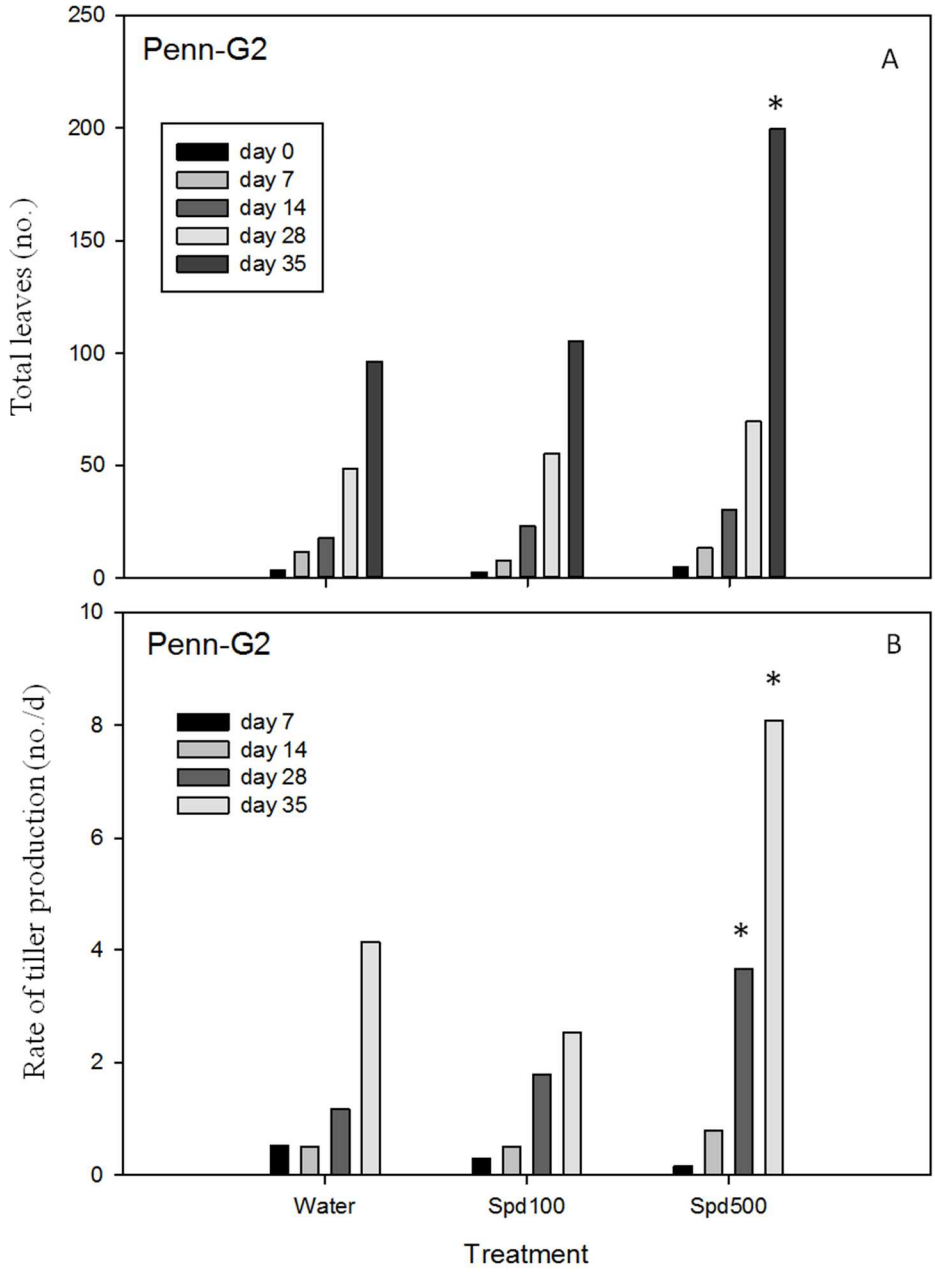

Figure 5: (A) Total leaves (no.) and (B) tillering rate of creeping bentgrass in a hydroponic growth chamber study in response to foliar treatment with water (control) or spermidine at 100 or 500 $\mu M \cdot L^{-1}$. Asterisks indicate statistically significant differences between chemical treatments on a given day of growth in the hydroponic system after chemical treatment. Asterisks were determined by Fisher's LSD tests (n = 4) at the $P \leq 0.05$ level.

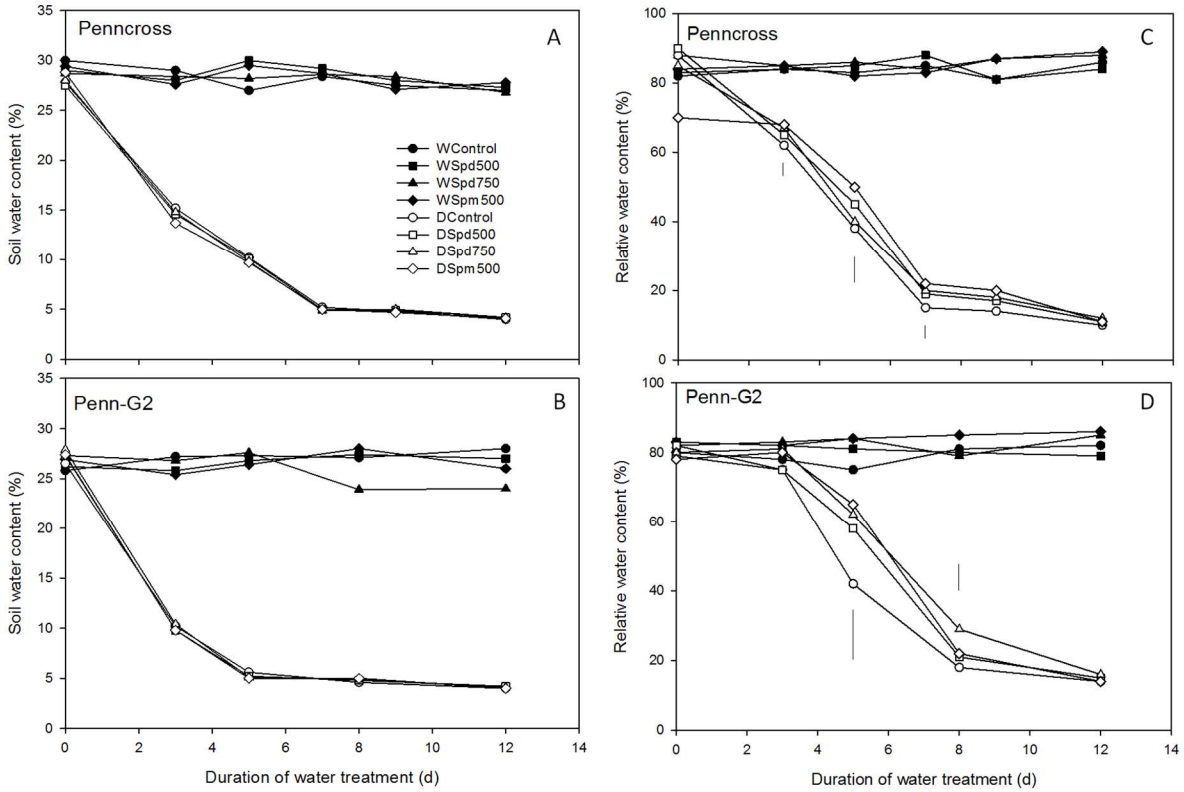

Figure 6: Effect of water treatment and chemical treatment on the soil volumetric water content (%) of (A) 'Penncross' and (B) 'Penn-G2'. Leaf relative water content (%) of creeping bentgrass in response to water and chemical treatment for (C) 'Penncross' and (D) 'Penn-G2'. LSD bars are present only on a given date where statistically significant differences were observed (n = 4) between chemical treatments ($P \leq 0.05$). W = watered, D = drought, Control = water plus Tween-20 (Sigma-Aldrich, St. Louis, MO), Spd = spermidine, Spm = spermine, 500 = 500 $\mu M \cdot L^{-1}$ treated, 750 = 750 $\mu M \cdot L^{-1}$ treated.

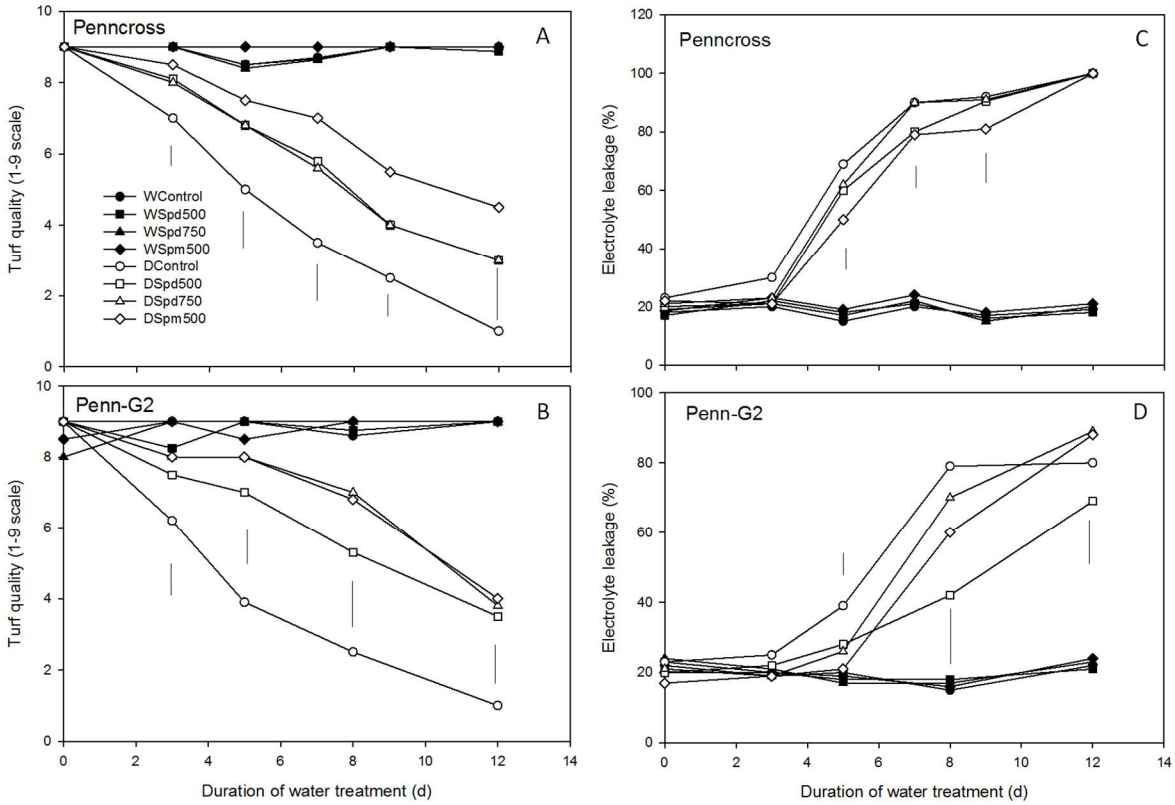

Figure 7: Effect of water treatment and chemical treatment on the turf quality rating (1-9 scale; 1= worst 9=best) of (A) 'Penncross' and (B) 'Penn-G2' creeping bentgrass. Electrolyte leakage of (C) 'Penncross' and (D) 'Penn-G2'. LSD bars are present only on a given date where statistically significant differences were observed (n = 4) between chemical treatments ($P \leq 0.05$). W = watered, D = drought, Control = water plus Tween-20 (Sigma-Aldrich, St. Louis, MO), Spd = spermidine, Spm = spermine, 500 = 500 $\mu M \cdot L^{-1}$ treated, 750 = 750 $\mu M \cdot L^{-1}$ treated.

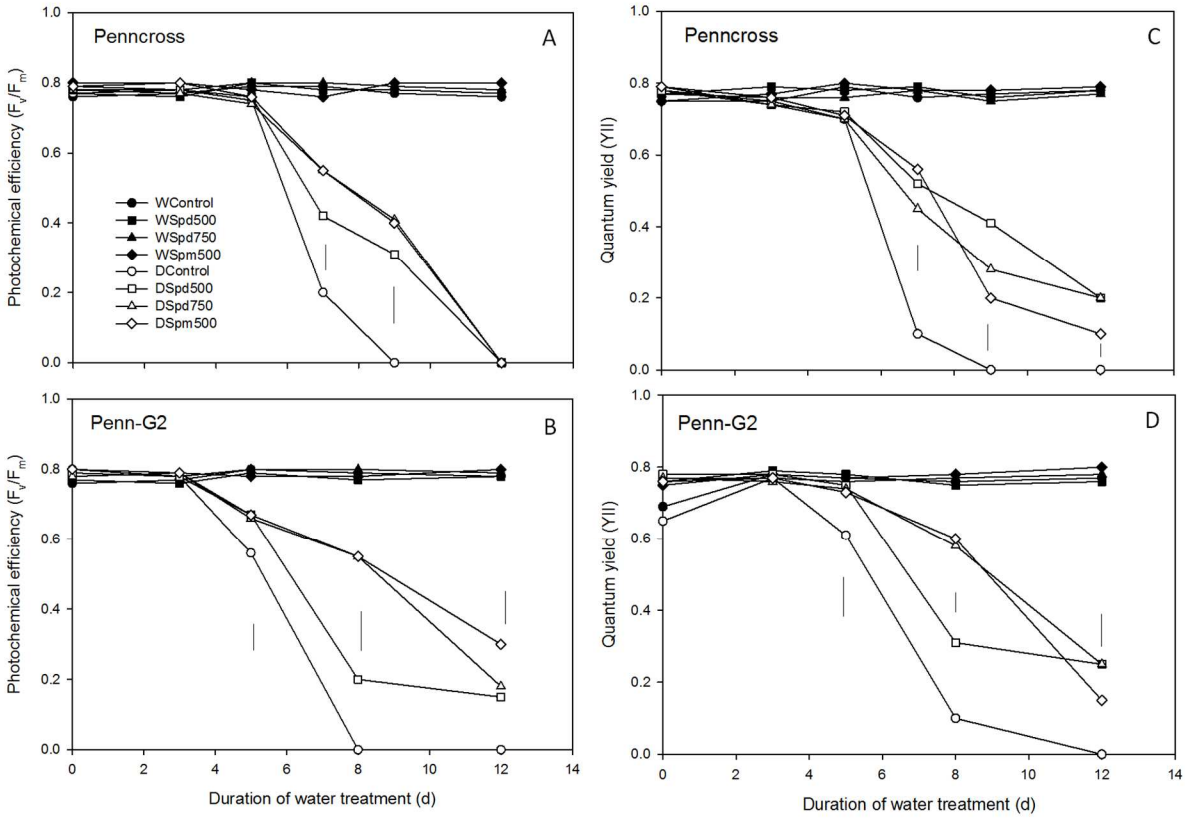

Figure 8: Effect of water and chemical treatment on photochemical attributes of creeping bentgrass plants. The photochemical efficiency (F_v/F_m) of (A) 'Penncross' and (B) 'Penn-G2' creeping bentgrass and quantum yield of photochemical energy conversion in PSII (YII) of (C) 'Penncross' and (D) 'Penn-G2'. LSD bars are present only on a given date where statistically significant differences were observed (n = 4) between chemical treatments ($P \leq 0.05$). W = watered, D = drought, Control = water plus Tween-20 (Sigma-Aldrich, St. Louis, MO), Spd = spermidine, Spm = spermine, 500 = 500 $\mu M \cdot L^{-1}$ treated, 750 = 750 $\mu M \cdot L^{-1}$ treated.

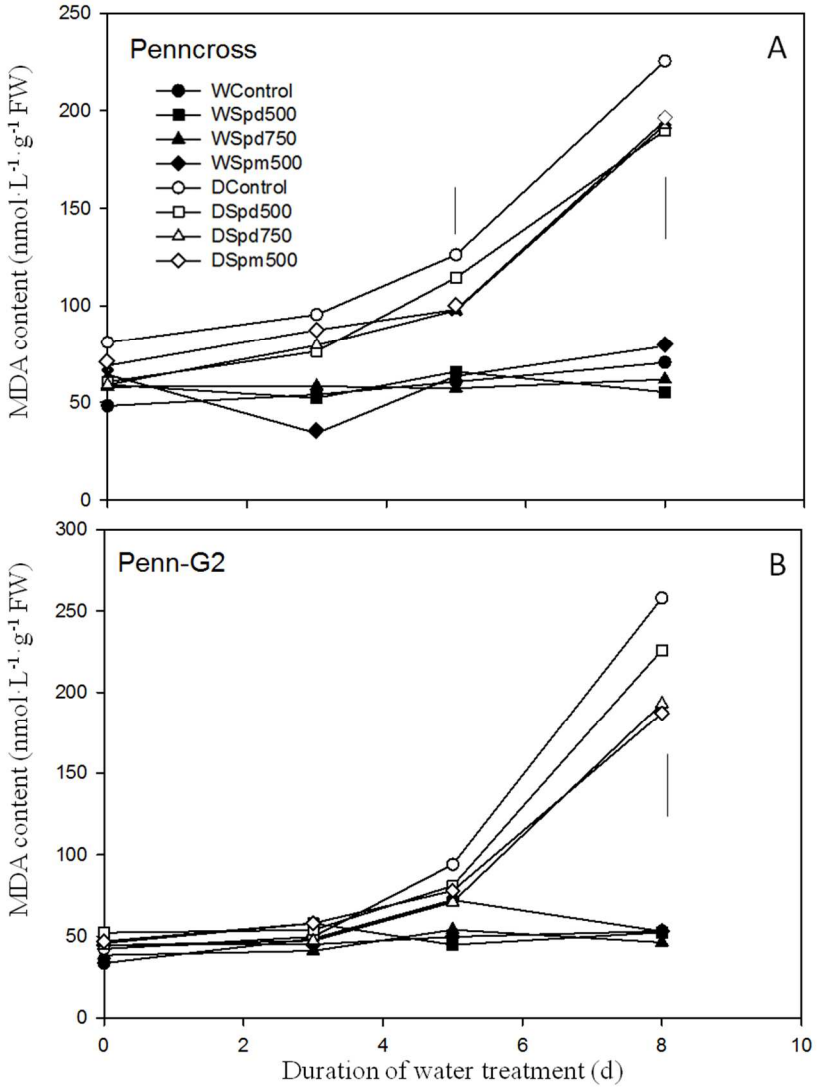

Figure 9: Leaf malondialdehyde content (MDA) of (A) 'Penncross' or (B) 'Penn-G2' creeping bentgrass exposed to water or chemical treatments. LSD bars are present only on a given date where statistically significant differences were observed (n = 4) between chemical treatments ($P \leq 0.05$). W = watered, D = drought, Control = water plus Tween-20 (Sigma-Aldrich, St. Louis, MO), Spd = spermidine, Spm = spermine, 500 = 500 $\mu M \cdot L^{-1}$ treated, 750 = 750 $\mu M \cdot L^{-1}$ treated.

Table 4: Analysis of variance (ANOVA) in type III error tests of fixed effects on various traits measured under different days after drought stress in 'Penncross' (0, 3, 5, 7, 9, and 12) and 'Penn-G2' (0, 3, 5, 8, and 12).

		Penncross						Pen-G2				
	Effect	**0**	**3**	**5**	**7**	**9**	**12**	**0**	**3**	**5**	**8**	**12**
	Drought	ns	*	*	*	*	*	ns	*	*	*	*
TDR	**Trt**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Drought *Trt	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Drought	ns	*	*	*	*	*	ns	ns	*	*	*
RWC	**Trt**	ns	*	*	*	ns	ns	ns	ns	*	*	ns
	Drought *Trt	ns	*	*	*	ns	ns	ns	ns	*	*	ns
	Drought	ns	*	*	*	*	*	ns	*	*	*	*
TQ	**Trt**	ns	*	*	*	*	*	ns	*	*	*	*
	Drought *Trt	ns	*	*	*	*	*	ns	*	*	*	*
	Drought	ns	ns	*	*	*	*	ns	ns	*	*	*
EL	**Trt**	ns	ns	*	*	*	ns	ns	ns	*	*	*
	Drought *Trt	ns	ns	*	*	*	ns	ns	ns	*	*	*
	Drought	ns	ns	ns	*	*	*	ns	ns	*	*	*
Fv/m	**Trt**	ns	ns	ns	*	*	ns	ns	ns	*	*	*
	Drought *Trt	ns	ns	ns	*	*	ns	ns	ns	*	*	*
	Drought	ns	ns	ns	*	*	*	ns	ns	*	*	*
Yield	**Trt**	ns	ns	ns	*	*	*	ns	ns	*	*	*
	Drought *Trt	ns	ns	ns	*	*	*	ns	ns	*	*	*
	Drought	ns	*	*	NA	*	NA	ns	ns	*	*	NA
MDA	**Trt**	ns	ns	*	NA	*	NA	ns	ns	ns	*	NA
	Drought *Trt	ns	ns	*	NA	*	NA	ns	ns	ns	*	NA

TDR indicates time domain reflectance for measuring soil volumetric water content; RWC indicates leaf relative water content; TQ indicates overall canopy turf quality rating based on the scale of 1 to 9; EL indicates percentage of leaf electrolyte leakage; Fv/m indicates dark adapted photochemical efficiency of photosystem II during photosynthesis process; Yield indicates light adapted photochemical yield of photosystem II during photosynthesis process; MDA indicates the leaf malondialdehyde content; NA indicates no data was collected on that date; Trt indicates the effect of Spd 500, Spd 750, and Spm 500 treatments; Drought indicates the effect of drought stress; Drought*Trt indicates the effect of interaction between drought and polyamine treatment. NS indicates a non-significant difference was detected; * represents significant effect at the level of 0.05.

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CHAPTER 4

TRANSCRIPTOME ANALYSIS OF CREEPING BENTGRASS EXPOSED TO DROUGHT STRESS AND POLYAMINE TREATMENT

Abstract

Creeping bentgrass is an important cool-season turfgrass species sensitive to drought. Treatment with polyamines (PAs) has been shown to improve drought tolerance; however, the mechanism is not yet fully understood. Therefore, this study aimed to evaluate transcriptome changes of creeping bentgrass in response to drought and exogenous spermidine (Spd) application using RNA sequencing (RNA-Seq). The high quality sequences were assembled and 18,682 out of 49,190 (38%) were detected as coding sequences. A total of 22% and 19% of genes were found to be either up- or down-regulated due to drought while 20% and 34% of genes were either up- or down- regulated in response to Spd application under drought conditions, respectively. Gene ontology (GO) and enrichment analysis were used to interpret the biological processes of transcripts and relative transcript abundance. Enriched or differentially expressed transcripts due to drought stress and/or Spd application were primarily associated with energy metabolism, transport, antioxidants, photosynthesis, signaling, stress defense, and cellular response to water deprivation. This research is the first to provide transcriptome data for creeping bentgrass under an abiotic stress using RNA-Seq analysis. Differentially expressed transcripts identified here could be further investigated for use as molecular markers or for functional analysis in responses to drought and Spd.

Keywords RNA-seq, turfgrass, plant abiotic stress, spermidine, polyamines, drought stress, transcriptome

Introduction

Creeping bentgrass (*Agrostis stolonifera*) is a perennial, cool-season turfgrass and forage species that is susceptible to various abiotic stresses, particularly drought stress. Drought stress causes a cascade of physiological changes in creeping bentgrass leading to inhibition of photosynthesis and disruption in numerous cellular components and processes (Merewitz and Huang, 2013). As a turfgrass under high input management conditions, creeping bentgrass primarily relies on drought tolerance mechanisms, as escape or avoidance mechanisms can be restricted by those turfgrass management practices. For instance, avoidance of drought by deep rooting is often restricted by severely low mowing height and the plant's natural regulation of the root to shoot ratio (Fry and Huang, 2004). Several tolerance mechanisms are thought to be of major importance in grass species including antioxidants and late embryogenesis abundant proteins (Shi et al., 2012; Hu et al., 2010; Jiang and Huang, 2002; Fu et al., 2007). Despite current knowledge of various tolerance pathways, major methods to improve drought tolerance of creeping bentgrass are still needed and many biochemical pathways associated with stress tolerance are poorly investigated in turf or forage grass species.

Polyamine (PA) biosynthesis has been shown to be involved in abiotic stress tolerance. PAs are aliphatic, amine compounds that play a major role in regulating numerous biological processes. Spermidine (Spd), spermine (Spm), and putrescine (Put) are three major PAs in plants, all of which are involved in signaling for cell growth, development, and stress responses (Gill and Tuteja, 2010). Exogenous PA application plays a protective role in reducing drought stress symptoms in various plant species. In creeping bentgrass, pretreatment with Spd improved photochemical efficiency during drought and reduced lipid

peroxidation (Shukla et al., 2015). Spm application enhanced antioxidant enzyme activities in creeping bentgrass during drought (Li et al., 2015a). Spm also alleviated drought stress of white clover (*Trifolium repens*) by increasing production of sucrose, fructose, and dehydrins (Li et al., 2015b). PAs may be involved in biosynthesis of auxin, abscisic acid (ABA), ethylene, and their transcription factors as well as cross-talking with reactive oxygen species (ROS) in Arabidopsis via over expressed endogenous Put and Spm (Marco et al., 2011). Our study takes a chemical priming approach to determine the effects of PA on the transcriptome changes for drought tolerance. How PAs may be associated with lipid peroxidation, antioxidant activities, carbohydrates, or other tolerance mechanisms has not yet been fully elucidated. Transcriptome analysis of creeping bentgrass treated with Spd will improve our understanding of the gene changes associated with PA pre-treatment during drought stress.

Creeping bentgrass is an allotetraploid species (2n = 4x =28) comprised of two A_2A_2 and A_3A_3 subgenomes (Chakraborty et al., 2005; Araneda et al., 2013). Heterozygosity is often problematic for transcriptome studies using hybridization based techniques. The vegetative samples we used here for RNA-Seq do not rely on hybridization and did not go through meiosis. This type of tissue is more reliable for plant species with complex genomes (Wang et al., 2009). Expressed sequence tags (ESTs) are available in the NCBI database, which have largely been generated for marker development and molecular map construction (Golembiewski et al., 1997; Vergara and Bughrara, 2003, 2004; Chakraborty et al., 2005, 2006). Currently, approximately 21, 545 ESTs (as of Jan 2017) are in the NCBI EST database, of which only 132 ESTs are associated with research aimed to evaluate creeping bentgrass for drought responses. A greater number of ESTs for creeping bentgrass for drought stress responses are needed in the database to serve as a resource for turf or forage grass scientists.

Gene expression changes on a whole transcriptome level associated with drought stress or drought protective compounds of turfgrass species have not been well-studied. RNA-Seq technology has been used in many other plant species and is powerful for plants that are not model species, have complex genomes, or do not have a fully sequenced genome (Wang et al., 2009). Some examples include sweet potato (*Ipomoea batatas*; Wang et al., 2010), watermelon (*Citrullus lanatus*; Guo et al., 2011), and white lupin (*Lupinus albus*; O'Rourke et al., 2013). So far, RNA-Seq has been used in turfgrass species for a better understanding of fungal pathogen interactions with creeping bentgrass (Orshinsky et al., 2012), morphological attributes of Kentucky bluegrass (*Poa pratensis*; Gan et al., 2016) and salt stress of Kentucky bluegrass (Bushman et al., 2016). To our knowledge, this is the first report of leaf transcriptome analysis by RNA-seq technology in creeping bentgrass subjected to an abiotic stress. Additionally, little information is available for transcriptome changes in response to PA application in crop species. Therefore, this work will serve as a valuable resource for future studies to improve drought tolerance in economically important turfgrass species and for better understanding the role of PAs in drought tolerance. The objectives of the study were to perform transcriptomic analysis by RNA-Seq to detect DE genes involved in creeping bentgrass under drought stress and PA application and to better interpret their biological meanings using gene ontology and enrichment analysis.

Materials and methods

Plant materials and growth conditions

The plants, experimental conditions, and treatments utilized for this study are described in more detail in Shukla et al. (2015). Briefly, creeping bentgrass 'Penn-G2' plants were pretreated with 500 $\mu M \cdot L^{-1}$ Spd. Experimental treatments included well-watered control

plants without Spd treatment (WC), well-watered plants with Spd treatment (WS), drought control plants without Spd treatment (DC), and drought-stressed plants with Spd treatment (DS). One day after, Spd treatment, half of the plants were subjected to drought stress with full water withholding for 12 d, which resulted in the soil water content (SWC) reaching 5%. Well-watered plants were maintained at approximately 25 to 28% SWC. Leaf tissue from each treatment was collected on 5 d of drought and was used for RNA-Seq analysis.

RNA isolation and cDNA synthesis

Plants were sampled for RNA-Seq analysis after 5 d of drought stress at the same level of SWC (5%), where Spd treated plants had a significantly greater photochemical efficiency. A total of 30 mg of frozen leaf tissue was homogenized in liquid nitrogen and total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA), as directed by the manufacturer's instruction. Any contaminated genomic DNA was removed by using RNase free DNase set (Qiagen). RNA quality was determined on a bioanalyzer (2100; Agilent Technologies, Santa Clara, CA). RNA concentration was quantified using a nanodrop (Thermal Scientific, Wilmington, DE). RNA (1µg) was used for cDNA synthesis using iScript^{TM} cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instruction.

cDNA sequencing and assembly

The cDNA samples were divided into four treatment groups with their biological replications: watered controls (WC1, WC2, WC3, and WC4), watered plants treated with Spd (WS1, WS2, WS3, and WS4), drought stress without Spd (DC2, DC3, and DC4), and drought stressed plants treated with Spd (DS1, DS2, DS3, and DS4). A total of 15 cDNA samples were used for library preparation and subjected to cDNA sequencing (Illumina HiSeq 2500, San Diego, CA). Illumina TruSeq RNA library preparation and quality control, Illumina HiSeq 2500

Rapid 2x150-bp sequencing, transcriptome quality control, assembly, alignment, scaffolding, and annotation were performed at the genomics core facility of Michigan State University (East Lansing, MI). Two lanes of sequencing were used to generate 260-300 million read pairs which provided coverage of approximately 15 times of the creeping bentgrass genome that is approximately 2800 Mbp (Xing et al., 2009). This provided good coverage for both de novo assembly (Table 7) and DE gene analysis.

Data quality control was performed by removing library adapter sequences, random hexamer priming bases, and low quality base calls. De novo transcriptome assembly was carried out with Trinity assembler (R20140413p; Grabherr et al., 2011). Input reads were normalized as fragments per kilobase of transcript per million mapped reads (FPKM) to reduce bias from highly abundant transcripts reads. Initial output of Trinity was a set of approximately 500,000 contigs (data not shown). All input reads were aligned to this set of contigs to produce an abundance estimation of each using Bowtie in Trinity. Contigs with extremely low numbers of reads mapped to them suggested they were artifacts and were filtered out. The N50 value, based on the longest isoform per 'gene', was 1562 bp.

Differentially expressed gene analysis

Pairwise analysis of DE genes was conducted using the Trinity toolset in Bioconductor package (Gentleman et al., 2004). Sample DC4 was excluded from further analysis because its expression pattern was more similar to the DS samples than the other two DC replicates based on initial sample correlation analysis (Figure 10 and 11). Other than this sample, the correlation analysis revealed consistency among biological replicates. All input reads were aligned to the set of filtered transcripts with Bowtie in Trinity and the abundance of each was estimated with RNA-Seq expectation-maximization (RSEM) which computes 'gene-level'

estimates as a proxy for the gene (Li and Dewey, 2011; Haas et al., 2013). In order to compare expression level of DE genes across treatments, each pair of DE genes was analyzed in turn (WC vs WS, DC vs DS, WC vs DC, WS vs DC, and WS vs DS) using EdgeR (version 2.14; Robinson et al., 2010). A web-based tool called Vennt (version 0.8.1; Powell, 2014) was utilized for examining lists of DE genes that are either up- or down-regulated defined at a false discovery rate threshold (FDR) of 0.001 and $\log_2$ fold change larger than 2.0.

Annotation analysis

Trinity filtered contigs (80,996) were subjected to a process that detects coding sequences (CDS) using Transdecoder in Trinity and those CDS were blasted using BLASTX against the SwissProt reference protein database (UniProt, 2014) to identify known proteins for functional annotation. Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway functional enrichment analyses were performed using Fisher's exact test module in Blast2GO software (version 2.7.2; Bluthgen et al., 2005; Conesa et al., 2005). The blast expectation value (E-value) was 0.001 and the highest scoring paring value was 33. GO is widely used to categorize over-represented genes for biological processes using annotation standards based on the top BLAST hits and identified domains (Stevens et al., 2000; Young et al., 2010). Enriched transcripts represented by number of tested sequences over reference sequences were provided where they were identified. DE genes were constructed in a heatmap using R package (Rx64, 3.3.2.3). Biological process (BP), molecular function, and cellular components were produced from GO where one or more levels of assemblies in molecular functions are used to describe a BP (Stevens et al., 2008). KEGG pathway provides a comprehensive way of interpreting the network of high throughput sequence data that is complementary to the currently published molecular biology where enzymes that encoded by

genes in the genome are reconstructed into a diagram by direct mapping of the GO with their enzyme codes for particular biochemical pathway (Ogata et al., 1999; Kanehisa and Goto, 2000; Conesa and Gotz 2008; Kanehisa et al., 2014). All genes detected in the KEGG pathways exposed to drought and Spd were summarized in a diagram.

qRT-PCR confirmation

cDNA was synthesized from the same RNA samples that were used in RNA-Seq analysis for qRT-PCR analysis. Fifty-four primer pairs were designed from RNA-seq sequences in TaqMan® MGB Quantification method (Primer Express 3.0; Table 6). Twenty out of 54 primer pairs were screened, selected, and subjected to qRT-PCR. PCR reactions were conducted using 2X Power SYBR® Green PCR Master Mix (Life Technologies Inc, Carlsbad, CA) in a fast real-time PCR system (7900 HT; Applied Biosystems, Foster City, CA). Actin was used as reference gene for data normalization.

Statistical analysis and data availability

Statistical analysis of RNA-Seq results was described above. For qRT-PCR, data analysis was performed using comparative cycles of threshold (CT) method to calculate the fold changes of each gene. The correlation between RNA-Seq results and qRT-PCR expression was analyzed using PROC COR procedure in SAS (SAS® 9.4 for Windows, Cary, NC) to get Pearson's correlation coefficients. The raw cDNA reads were deposited in sequence read archives (SRA). The transcriptome shotgun assembly project has been deposited at GenBank under the accession GEUC00000000. The version described in this paper is the first version, GEUC01000000. In order to meet the guidelines set by the transcriptome shotgun assembly from NCBI, two contigs required trimming due to adapter contamination. The first 25 bases of the 5' end of c211554_g1_i7 were trimmed off. The last 38 bases of the 3' end of

c206014_g1_i3 were trimmed off. Gene expression analysis was not updated to reflect this change as the modification to these contigs had a marginal effect on the FPKM values computed and, to an even lesser extent, the differential gene expression.

Results

Sequence assembly

All reads were aligned to the sets of filtered transcripts (80,996) and alignment efficiency was calculated as a percentage for number of aligned reads over the filtered read pairs in assembly (Table 7). All alignment efficiencies were about 60% while DS2 had 74% of its input reads aligned. In model organisms such as Arabidopsis, researchers may obtain higher percentage of alignments mapped, but for a de novo assembly without reference genome for which we have created a reference transcriptome; these results are to be expected. Open reading frames (ORF) and translated peptide sequences were identified in Trinity in which showed 80,996 transcripts as coming from 51,571 genes. Of 80,996 transcripts, 49,190 ORFs meeting minimal criteria were identified. 18,682 of these were complete coding sequences (Accessions deposited in GenBank).

Differential expression and gene ontology

A total of 9,109 transcripts were identified as DE genes in one or more of the pairwise comparisons (Figure 12). Under well-watered conditions, the addition of spermidine (Spd) had little effect on transcription. This was also made clear from the heatmap of gene expression for all DE genes where the distance between these two groups (WC vs WS) was not significantly different from the distance between replicates within each group (Figure 11). The heatmap also indicates the overall effect on drought stress on transcription and allows for visualization of how Spd moderated the effects of drought stress on the transcriptome. A large

change of the transcriptome occurred in creeping bentgrass in response to drought stress. A total of 6,504 genes were either up- or down-regulated when comparing well-watered to drought stressed plants (Figure 12). Gene ontology (GO) and enrichment analysis identified 741 biological processes, 197 cellular components, and 334 molecular functions. Only the GOs most relevant to drought stress and Spd application are focused on in the discussion.

Differentially expressed genes due to drought stress

When compared between drought and well-watered samples (WC vs DC), 22% (860 out of 3936) transcripts were up-regulated while 500 genes out of 2564 (20%) were down-regulated (Figure 13). In response to drought, up-regulated DE genes were identified to encode heat stress transcription factor (5.9 fold) in stress response (GO: 0006950; FDR<0.0001), probable peroxygenase 4 (10.1 fold) associated with response to abscisic acid (ABA; GO: 0009737; FDR<0.0001), and tryptophan synthase beta chain 2 (5.7 fold) in oxidative stress (GO: 0006979; FDR<0.0001; Figure 14). In addition, genes encoding aminocyclopropane-1-carboxylate oxidase 2 (2.5 fold) associated with ethylene biosynthesis (GO: 0009693; FDR=0.0074) were identified to be up-regulated for hormone related leaf senescence due to drought stress (Figure 16).

Enriched transcripts associated with amino acid biosynthesis were identified due to drought. Up-regulation of a DE gene encoding a probable electron transfer flavoprotein-quinone oxidoreductase (3.2 fold) in nitrogen compound metabolic process (GO: 0006807; FDR<0.0001) was detected. Additionally, up-regulation occurred for DE genes encoding homocysteine methyltransferase (3.4 fold) in methionine biosynthesis (GO: 0009086; FDR= 0.0105), acetylornithine deacetylase (3.8 fold) in arginine biosynthesis (GO: 0006526; FDR= 0.0261), probable serine acetyltransferase (3.6 fold) in cysteine biosynthesis from serine (GO:

0006535; FDR<0.0001), and 3-isopropylmalate dehydratase large subunit (5.1 fold) in leucine biosynthesis (GO: 0009098; FDR =0.0466). A DE gene was also detected in Spd biosynthesis (GO: 0008295; FDR= 0.0007; Figure 16, 17, 18).

Enriched transcripts associated with sugar metabolism were detected. When compared to well-watered plants, drought caused an up-regulation of beta-amylase transcripts (7.6 fold) involved in water deprivation (GO: 0009414; FDR<0.0001). Up-regulation was also identified for glucose-6-phosphate 1-dehydrogenase transcripts (4.9 fold) involved in the pentose-phosphate shunt (GO: 0006098; FDR=0.0442; Figure 14, 19).

Differentially expressed genes due to Spd application under watered conditions

When samples without Spd treatment were compared to samples treated with Spd under well-watered conditions (WC vs WS), only 37 out of 9109 transcripts were differentially expressed. This indicates that Spd did not play a major role regulating transcription under watered conditions in creeping bentgrass (data not shown).

Differentially expressed genes due to drought of spermidine treatment

Comparing plants treated with Spd under well-watered to drought stressed conditions (WS vs DS), 19% (727 out of 3803) of the transcripts were up-regulated and 34% (1086 out of 3154) were down-regulated (Figure 13). These regulated transcripts were enriched in response to drought stress and were involved in many biochemical processes. For instance, Spd application caused up-regulation of genes associated with photosynthesis under drought, such as a gene encoding photosystem II 10 kDa polypeptide (3.1 fold) involved in photosynthesis (GO: 0015979; FDR<0.0001; Figure 14), ribulose bisphosphate carboxylase small chain clone 512 (GO:0019253; FDR<0.0001; -9.9 fold), and phosphoenolpyruvate carboxylase (PEPC; 4.2 fold) in carbon fixation (GO:0015977; FDR<0.0001) (Figure 14 and 20).

Sugar metabolism related transcripts enrichment was affiliated with Spd application under drought stress. When comparing drought and well-watered plants, up-regulation was detected for DE genes encoding beta-amylase 1 (7.1 fold) in response to water deprivation (GO: 0009414; FDR<0.0001), sucrose synthase (SS) 4 (7.5 fold) and SS4 (3 fold) involved in starch (GO:0005982; FDR<0.0001) and sucrose (GO:0005985; FDR=0.00017) metabolism, respectively. Expression of a gene encoding galactinol-sucrose galactosyltransferase 1 (-4.5 fold) in carbohydrate metabolism (GO:0005975; FDR<0.0001) was down-regulated (Figure 14 and 21).

Transporter associated genes were detected in drought and Spd treated plants. For instance, down-regulation occurred for a gene encoding a high affinity nitrate transporter (-4.5 fold) involved in nitrate assimilation (GO:0042128; FDR=0.004), and a calcium-binding mitochondrial carrier protein, SCaMC-1 (-5.1 fold), involved in transmembrane transport (GO:0055085; FDR<0.0001). Up-regulation for a gene encoding a bidirectional sugar transporter SWEET15 (2.9 fold), within the GO category of cellular response to stimulus (GO:0071215; FDR<0.0001), was also found (Figure 14).

Enrichment for transcripts associated with signaling was also detected. When comparing drought and well-watered plants, genes encoding a gibberellin (GA) regulated protein 2 (2.1 fold) involved in GA mediated signaling pathway (GO:0009740; FDR<0.0001) and CHD3-type chromatin-remodeling factor PICKLE (3.4 fold) involved in cytokinin-activated signaling pathway (GO:0009736; FDR=0.018) were up-regulated (Figure 14). Expression of a gene encoding an ethylene-responsive transcription factor, ERF054, (-4.5 fold) that is involved in the ethylene-activated signaling pathway (GO:0009873; FDR<0.0001) was down-regulated (Figure 14).

DE genes related to the antioxidant system were detected. For instance, a gene encoding a cationic peroxidase SPC4 (2.2 fold) involved in hydrogen peroxide catabolic process (GO:0042744; FDR<0.0001) was up-regulated due to Spd treatment. Expression of a gene encoding a glutathione S-transferase GSTU1 (-2.8 fold), involved in cellular response to water deprivation processes (GO:0042631; FDR=0.0125), was down-regulated due to Spd treatment (Figure 14).

qRT-PCR validation of RNA-Seq results

A total of 20 genes used for qRT-PCR had 90% ± 10% of amplification efficiencies with a single dissociation peak and linearity between target cDNA and Ct values. These genes used for qRT-PCR were all consistent with the RNA-seq results (Pearson's $r = 0.83$, $P < 0.001$; Figure 15).

Discussion

Due to the widespread availability of transcriptome data associated with drought stress in model and crop species such as in Arabidopsis and maize (*Zea mays*; Deyholos, 2010), the following includes a brief discussion of gene changes solely due to drought stress (WC vs DC) but is primarily focused on PA effects on drought tolerance. A discussion of other relevant and interesting gene changes that may be specific for creeping bentgrass is also provided. Major changes in transcriptome due to Spd treatment under watered conditions (WC vs WS) were not detected; however, to avoid negating any differences due to Spd treatment under watered conditions, this discussion will primarily focus on comparing WS to DS instead of WC to DS, in order to not confound DEG results revealed and for a more concise discussion of the results. It is also worthy to note that often transcriptome changes are not always correlated to changes in protein expression such as the discrepancies found between

microarray and protein profiling of salt stress of Arabidopsis (Jiang et al., 2007). Further experimental evidence is needed to confirm the fate of the genes identified here; however, this work provides a good reference for genes of interest associated with PAs and drought stress.

Genes differentially expressed due to drought stress

Drought-induced physiological changes are a result of numerous gene expression changes that act to alter biochemical processes to escape, avoid, or tolerate drought stress such as photosynthesis, respiration, sugar metabolism, defense pathways, and hormone signaling (Merewitz et al., 2011; Huang et al., 2014). ABA and ethylene are the most closely associated hormones with drought stress perception and signaling (Wilkinson and Davies, 2010). In this study, enriched transcripts associated with ethylene were found. Drought stress caused an up-regulation of genes encoding amino-cyclopropane-carboxylate oxidase (ACC oxidase; 2.4 fold) which converts ACC into ethylene (Miyazaki and Yang, 1987) and two ethylene transcription factors, APETALA2/ethylene response (RAP2-4; 3.3 fold) and ethylene response factor (ERF054; 5.6 fold). This is consistent with transcript enrichment of ethylene biosynthesis in response to drought in soybean (*Glycine max*; Arraes et al., 2015). In Arabidopsis, RAP2-4 mediates ethylene signaling pathways by constitutively binding to ethylene and dehydration responsive elements during drought (Lin et al., 2008). Similar to these findings, over expression of transcription factor (*ERF*) in wheat (*Triticum aestivum*; Rong et al., 2014) and Arabidopsis (Cheng et al., 2013) showed significantly higher drought and salt tolerance than wild type via accumulation of proline, maintaining redox homeostasis, reduced transpiration water loss, and lower stomatal conductance. Relatively little information regarding ethylene biosynthesis and drought stress is available for creeping bentgrass or other important turf or forage species; further investigation into ethylene responses during drought

stress and how ethylene relates to PAs may be warranted for improvement of creeping bentgrass performance under drought stress.

Drought induced an up-regulation (3.5 fold) of a gene encoding ABA biosynthesis in creeping bentgrass. Up-regulation of ABA biosynthesis gene expression is expected as it can trigger stomatal closure (Grondina et al., 2015; Tombesi et al., 2015). ABA can also regulate molecular chaperones (Campalans et al., 1999), which are a family of proteins that facilitate protein folding, reducing misfolding, stabilizing or maintaining the integrity of the cell membrane or enzymes, or preventing aggregation or disaggregation of proteins for normal function (Hart and Hayer-Hart, 2002). In creeping bentgrass, the expression of a gene encoding dehydrin RAB15 was up-regulated in response to drought stress. RAB15 is an ABA responsive dehydrin, which has chaperone-like functions to maintain the integrity of cell walls in wheat (King et al., 1992; Brinia et al., 2007) and reduce water loss in a drought tolerant Bermuda grass (*Cynodon dactylon*; Hu et al., 2010). Another gene encoding chaperone type protein, dehydrin COR410, was up-regulated (5.1 fold) by drought in creeping bentgrass. COR410 was initially identified in the plants under cold stress while it was also induced by ABA and drought (Choi et al., 1999). The drought induced expression of gene encoding COR410 is observed in wheat (Eini et al., 2013) and over expression of *COR410* protected cell membranes during cold stress of strawberry (*Fragaria* × *ananassa*; Houde et al., 2004). Thus, up-regulation of these ABA and drought induced molecular chaperones may be of critical importance for creeping bentgrass survival under drought stress.

Osmoprotectant production plays an important role in the response to drought. One of the amino acid, proline, acts as osmotic compatible solute (Zhang et al., 2009) and free radicals scavenger (Seki et al., 2007). A gene encoding pyrroline-5-carboxylate reductase

(P5CR) was up-regulated by 3.5 fold due to drought stress. P5CR is the rate limiting enzyme that catalyzes the conversion of δ^1-pyrroline-5-carboxylate to L-proline (Hare et al., 1999). In Arabidopsis, transcript induction of *P5CR* gene is associated with increased accumulation of proline after salt stress (Verbruggen et al., 1993). Site-directed mutation of *P5CR* gene feedback inhibitor showed more proline accumulation than the wild type in response to osmotic stress, which was associated with reduced malondialdehyde accumulation and osmotic stress tolerance (Hong et al., 2000). Similarly, a potassium channel gene KOR1 was up-regulated by 4.4 fold. Arabidopsis mutant for gene encoding inward-rectifying K^+ channel that uptakes K^+ showed less K^+ uptake and poor growth (Hirsch et al., 1998). Thus, up-regulated gene expression on biosynthesis of osmotic protectant and K^+ transporters may be associated with osmotic adjustment to contribute drought tolerance in creeping bentgrass.

Drought stress significantly induced transcript enrichment associated with amino acid biosynthesis (WC vs DC; Figure 16). For instance, GO categories for biosynthesis of methionine, cysteine, and leucine were enriched. More specifically, gene expression for 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase which functions for methionine (Met) formation (Ferla and Patrick, 2014) was up-regulated. In addition to be proteinogenic of these amino acids, they are also known to be involved in biosynthesis of other compounds or being associated with stress tolerance. Met serves as a fundamental precursor for S-Adenosyl-methionine (SAM) biosynthesis and controls ethylene and PAs biosynthesis (Roje, 2006). Although both ethylene and PAs are involved in drought stress tolerance in several crop species (Merewitz, 2016), the direct abiotic stress tolerance effect of methionine has not been documented. The increased transcripts in Met biosynthesis could be for increased need of Met for protein synthesis. Biosynthesis of cysteine from serine is

associated with nitrogen metabolism and stress defense (Hesse et al., 2004; Kopriva et al., 2012). In soybean, H_2O_2 regulates accumulation and phosphorylation of acetyltransferase to catalyze biosynthesis of cysteine from serine. Increased cysteine accumulation due to increased activity of acetyltransferase was positively correlated with induction of glutathione, which might indicate that cysteine plays a role under oxidative stress (Liu et al., 2006). The drought-induced enrichment of transcripts related to amino acid biosynthesis may be associated with stress defense or building stress defense proteins; however, further work on these amino acids would be required to draw such conclusions.

PA biosynthesis was also found to be affected by drought stress. Expression of genes encoding arginine decarboxylase (ADC) 1 (2.7 fold), one of the enzymes that catalyze Put formation (Feirer et al., 1984), and arginine biosynthesis were up-regulated. Additionally, a gene encoding S-adenosylmethionine decarboxylase which catalyzes Spm formation was up-regulated by 6.3 fold due to drought stress (Figure 16). Put and Spm improved drought tolerance in wheat via mediating ion channels and cross talk with other phytohormones (Liu et al., 2000; Du et al., 2015; Merewitz, 2016). PAs alleviated some drought stress symptoms in Bermuda grass (Chan and Shi, 2015) and creeping bentgrass (Shukla et al., 2015; Ma and Merewitz, 2016). Although Spd content accumulation in creeping bentgrass may not be a naturally occurring major salt tolerance mechanism (Ma and Merewitz, 2016), three genes associated with Put and Spm biosynthesis were affected by drought stress. Therefore, exploitation of this stress tolerance pathway, via exogenous application of Spd, may be a viable method to promote PA-induced stress tolerance in creeping bentgrass.

Spd effects on drought tolerance

Photosynthesis

Drought stress often causes photosynthesis rates to decrease due to stomatal closure, reduced sugar demands due to growth cessation, and metabolic damage of photosynthetic apparati (Chaves et al., 2009). The ability of plants to maintain photochemical health and efficiency under stressed conditions is a major drought tolerance mechanism (Wingler et al., 1999). Exogenously applied Spd in creeping bentgrass helped maintain photochemical health during drought stress compared to control plants (Shukla et al., 2015). Photochemical efficiency of the light reactions of photosynthesis is often measured by the health of photosystem II (PSII) and PSII can be damaged by drought stress (Cornic and Fresneau, 2002). Spd application significantly up-regulated (3.1 fold) expression of gene encoding a 10 kDa PSII polypeptide under drought conditions (Figure 16). Direct effect of PAs on PSII proteins revealed that low concentration (<1 mM) of Spm could bind to PSII membranes to maintain its integrity and improve photosynthetic function under stress (Hamdani et al., 2011). Treatment with 50 μM Spd has been shown to increase Fv/Fm, which indicates the enhancement of PSII function (Ioannidis and Kotzabasis, 2007). Up-regulation of PSII polypeptides, which could replace damaged subunits, and possible direct protection of PSII complexes by PA treatment may benefit plants under drought stress.

Exogenously applied Spd under drought stress also appears to regulate transcripts associated with the Calvin-Benson Cycle (CBC) of photosynthesis. Expression of seven genes encoding ribulose bisphosphate carboxylase (Rubisco) small chain clone 512 (Rubisco-small) were down-regulated (-9.9 fold) in plants treated with Spd under drought condition while no regulation was found for these genes due to drought. Rubisco is comprised of large and small

subunits and is a rate limiting enzyme in photosynthesis. Rubisco small subunits stabilize the large subunit to influence holoenzyme activity and substrate affinities (Andrews, 1988; Gutteridge, 1991). Genetically engineered cyanobacteria containing genes encoding a Rubisco small subunit increased the CO_2/O_2 catalytic efficiency and specificity compared to non-transgenic cyanobacteria (Harpel et al., 1995; Kostov et al., 1997) and miRNA based gene silencing of Rubisco small subunits decreased Rubisco activity in drought resistant *Physcomitrella patens* (Wan et al., 2011). Photorespiration activity is primarily determined by RuBP regeneration rate, CO_2/O_2 ratio in the chloroplast, as well as the amount and kinetics of the Rubisco holoenzyme (Ogren, 1984; Douce and Neuburger, 1999). Spd induced down-regulation of Rubisco small subunits could decrease Rubisco activity and could reduce both photosynthetic and photorespiratory processes in creeping bentgrass. This could reduce energy expenditure associated with these processes during drought stress. Further research that may directly elucidate the effects of PAs on these processes is needed.

Expression of a gene encoding phosphoenolpyruvate carboxylase (PEPC) was up-regulated (4.2 fold) in response to Spd treatment under drought. PEPC is most well-known for the carboxylation reaction in C4 and crassulacean acid metabolism (CAM) photosynthesis (Britto and Kronzucker, 2005; Cousins et al., 2007). Over-expression of the maize *PEPC* gene in rice exhibited a higher RWC and higher chlorophyll content than the wild type plants, which could indicate greater drought tolerance (Shen et al., 2015; Qian et al., 2015). In C3 creeping bentgrass, exogenously applied Spd under drought stress appears to regulate transcripts associated with photosynthesis (Figure 16). Malate formed through PEPC might be involved in providing metabolic intermediates from the tricarboxylic acid cycle for stress survival (Araujo et al., 2012) and CO_2 fixation that formed via PEPC could contribute to

Calvin-Benson cycle due to stomata closure. Whether this directly correlates to photosynthesis rates under drought may deserve further investigation.

Sugar metabolism

Spd treatment may also influence other carbon relations and sugar metabolism in creeping bentgrass exposed to drought stress based on transcript enrichment (345/160). Expression of a gene encoding beta-amylase which functions to degrade starch to form glucose and maltose (Critchley et al., 2001) was up-regulated based on the comparison of WS vs DS (7.1 fold). Due to up-regulation of this starch degradation process, Spd application may increase the starch to glucose conversion process and have an effect to allocate sugar energy during drought stress.

Up-regulation was detected for genes encoding sucrose synthase (SS) 1 (3 fold) and SS4 (7.5 fold) due to drought under Spd treatment (WS vs DS). SS catalyzes the reversible reaction of sucrose formation and degradation, using glucose and fructose as substrates (Fu et al., 2010). Conversely, protein expression of SS was decreased in Spm treated white clover under drought (Li et al., 2015b). Increased *SS* expression and SS content was also identified in transgenic plants with increased cytokine content, which were more drought tolerant than non-transgenic plants (Merewitz et al., 2012; Reguera et al., 2013). Up-regulation of *SS* under drought might be activated for supplying metabolic intermediates, for respiration, or for regulating osmotic potential under drought stress; however, a better understanding of PA regulation of SS may be needed.

Similar to the regulation of *SS*, enriched transcripts (1979/1278) were identified in sucrose metabolism in response to Spd treatment. Except for the up-regulation of a gene encoding galactinol-sucrose galactosyltransferase (GSG; 5.7-fold) due to drought (WC vs

DC), expression for this process was down-regulated in Spd treated plants under comparison of WS vs DS (-4.5 fold). GSG catalyzes the biosynthesis of galactinol, raffinose, and other oligosaccharides (Nishizawa et al., 2008). Down-regulation of *GSG* in Spd treated plants might indicate that PA treatment is altering sugar metabolism in plants. Little information is available regarding the effects of PAs on sugar relations in the plant; further research on this may be desirable.

Transporters

In addition to sugar metabolism, transcript enrichment (185/110) was found to be associated with carbohydrate transport in plant cells due to Spd treatment. Carbohydrate transporters play a role in photosynthate allocation; however, not much information is available about relationship between PA and sugar transporters. We found expression of a gene encoding bidirectional SWEET transporter was up-regulated in comparisons of WC vs DC (9.3 fold) and in WS vs DS (2.9 -fold). SWEET transporters were phloem sugar loading transporters which regulate sugar movement and allocation in response to abiotic stress, plant growth, and development (Chen et al., 2012; Jarzyniak and Jasiński, 2014). If Spd treatment can enhance photosynthetic health, it makes sense that transport of those sugars would be differential compared to plants not treated with Spd. A gene encoding plastidic glucose transporter 3 was up-regulated in Spd treated plants by 6.3-fold. Starch formed during photosynthesis can break down to form glucose to go towards growth, cellular maintenance, or stress survival. Glucose transporters play an important role in the translocation of glucose from a chloroplast and within a cell (Weber et al., 2000). Bourque et al. (Bourque et al., 2002) showed glucose transporter in tobacco is involved in programmed cell death that induced by biotic stress to minimize the stress damage. During drought stress, a more effective synthesis and movement

of sugars within plant cells could be a major drought tolerance mechanism due to limited carbon acquisition due to stomatal closure and depletion of carbohydrates via respiratory processes. Further research into the effects of PAs and the effects of drought tolerance on sugar transporters is needed.

Membrane transport processes can become active for transporting organic or inorganic ions or amino acids to cope with adverse growing environment such as for osmotic adjustment (Jarzyniak and Jasiński, 2014). Expression patterns for amino acid transporters in plants can vary under different growing environments. Enriched transcript (10/1) was found in response to Spd application under drought. Expression of DE genes encoding an amino acid permease (AAP) 3 were up-regulated 4.2 fold in the comparisons of WS vs DS. AAP is a family of amino acid transporters that preferentially transport glutamine, asparagine, glutamate, and neutral amino acids into plant cells (Fischer et al., 2002). Glutamate acts as a precursor of proline biosynthesis to serve as an osmolyte (Verslues and Sharma, 2010). Another study also demonstrated that accumulation of glutamine and asparagine in younger leaves of resurrection plant (*Sporobolus stapfianus*) was more desiccation tolerant than the older leaves (Martinelli et al., 2007). It is possible that up-regulation of these amino acid transporters might be involved in amino acid-based osmotic regulation under drought in response to Spd treatment. Furthermore, we detected enriched transcript (21/8) for other amino acid transporters. Expression of gene encoding lysine histidine transporter (LHT) was down regulated (-8.7) in both drought stressed (WC vs DC) and drought Spd treated plants (WS vs DS). LHT is an amino acid permease homolog which serves as an amino acid selective transporter, especially lysine and histidine. Transcripts encoding LHT is greatly up-regulated by ABA, amino acid, JA, and SA in ginseng for dealing with environmental stresses

(Zhang et al., 2013) and mediating nitrogen use efficiency (Hirner et al., 2006). Lysine is also found to be as source of energy when carbon is depleted under drought stress in Arabidopsis and tobacco (Bunsupa et al., 2012). Two genes encoding lysine catabolism enzymes under osmotic stress in rapeseed (*Brassica napus*) were identified to be coexisting with proline biosynthesis gene which implies that lysine may play a role in withstanding osmotic stress (Moulin et al., 2000). Therefore, reduced compartmentalization of useful amino acids and activation of selective amino acid transporters for signaling, energy, or stress protection in response to Spd treatment may play a role in Spd-induced drought protection of creeping bentgrass.

Members of ATP-binding cassette (ABC) family are membrane-bound proteins that participate in transporting wide range of molecules with several categories (eg. A to H; Verrier et al., 2008). A gene encoding one member of the ABC transporters, ABCG protein, was down-regulated in Spd treated plants by 5.1-fold while no regulation was found due to drought. ABCG is known to be involved in transporting ABA to regulate stomata conductance (Jarzyniak and Jasiński, 2014), cytokines to mediate growth (Borghi et al., 2015), and cuticle precursors into the apoplast for cuticular wax deposition (Pighin et al., 2004). Down-regulation of an ABC transporter detected only in Spd treated plants, demonstrates there may be a regulatory effect of Spd on ABCG transporters. ABC transporters, such as ABCG, are driven by ATP hydrolysis acting as exporters and importers which is energy expensive (Kang et al., 2011). Spd treated plants could benefit from less energy expenditure by a reduced number of these transporters; however, it is not clear how PA-induced regulation of ABC transporters may play a role in the drought response of creeping bentgrass.

Transporters are also involved in mediating signaling transducers. One example is that spikes of free calcium ions decoded by calcium binding proteins involves in calcium signaling and leads to a signal amplification or physiological change to adapt to changing environmental conditions (Stael et al., 2011). Enrichment (543/341) of calcium binding proteins was identified. Expression of gene encoding calcium-binding mitochondrial carrier protein SCaMC-1 was up-regulated by 5.1-fold when compared to plants treated with Spd under drought (WS vs DS). This transporter functions as an ATP importer in the mitochondria and S-Adenosyl methionine transporter in plastid to cope with stress in Arabidopsis (Stael et al., 2011). It also acts as cell traffic mediator in sweet orange seedlings (*Citrus sinensis*) under boron deficiency (Lu et al., 2015). Up-regulation of these calcium involved transporters might present another strategy of Spd mediated drought tolerant by triggering calcium induced signaling pathways.

Signaling processes

Plants undergo a series of signaling transduction via various interactions among phytohormones and downstream signaling transducers for drought tolerance. A drought tolerant model revealed by the action of PA was proposed by Hatmi et al. (2015) in grapevine (*Vitis vinifera*) in which PA homeostasis was regulated to trigger downstream defense pathways through signal transduction. Pál et al. (2015) also indicated that PAs cross-talk with NO, H_2O_2, and Ca^{2+}, which mediate other phytohormones and signaling molecules to promote abiotic stress defenses. In response to Spd treatment, a DE gene in a calcium-mediated signaling pathway was down-regulated (-6.8 fold) when compared between drought Spd treated and well-watered plants (WS vs DS). Transgenic Arabidopsis lines with increased endogenous Put and Spm exhibited drought tolerance by means of cross-talk with ABA, Ca^{2+},

and other hormonal pathways (Marco et al., 2011). Over expressing *SAMDC*1 to increase endogenous Spm content in Arabidopsis induced up-regulation of a gene encoding an ABA biosynthesis gene (*NCED*), and those plants were more salt tolerant (Marco et al., 2011). The phenotype of hypersensitivity to salinity stress in Spm deficient Arabidopsis mutant (*acl5/spms*) is similar to Arabidopsis that over expresses a gene encoding a Ca^{2+}/H^{+} antiporter. This Spm mutant performs poorly in Ca^{2+} deficient media, which indicates the close relationship between Spm and Ca^{2+} (Yamaguchi et al., 2007).

Pottosin and Shabala (2014) further showed that Ca^{2+} influx across plasma membranes was induced by oxidation of PAs at the apoplast with Ca^{2+} as the second messenger to regulate stomata movement to promote drought tolerance. Here we found one DE gene associated with Ca^{2+} signaling and we found that it was down-regulated due to Spd treatment. As Ca^{2+} is involved in signaling a myriad of different processes in the plant in addition to stomatal regulation, it is unclear whether Spd may affect Ca^{2+} mediated signaling that is associated with drought tolerance in this study.

Cross-talk among the hormones ABA and ethylene and GA are known to play an important role in stress signaling (Achard et al., 2006). Limited information is available regarding how PAs may interact phytohormones metabolism to affect drought tolerance; in particular GA. Enriched transcripts (3595/2287) associated with GA signaling were detected in response to Spd application in creeping bentgrass. Expression of a gene encoding a GA regulated protein 2 was up-regulated (2.1-fold) when compared between drought and well-watered plants with Spd treatment (WS vs DS). Comparatively, the extent of up-regulation for this gene under drought (WC vs DC) was 7.5-fold. It is clear that there is a connection between PAs and GA signaling. Dwarfism caused by increased Put content in transgenic

Arabidopsis was rescued by exogenous GA application (Alcázar et al., 2005). Shukla et al. (2015) found that Spd treatment may promote creeping bentgrass tillering rates and leaf number compared with the non-Spd treated plants. It is not yet clear how GA signaling may be associated with PA function. Further evaluation of Spd regulation of GA mediated signaling for drought tolerance will enhance our understanding of GA and PAs in drought tolerance.

Stress defense

Antioxidants are a major part of the plant defense mechanisms under various environmental stresses since they scavenge ROS produced under stress to reduce damages to cellular constituents (Veljovic-Jovanovic et al., 2006). Effective regulation and maintenance of antioxidant systems can play a major role in the drought tolerance of creeping bentgrass and other perennial grass species (Merewitz et al. 2011; Shi et al., 2012; Jiang and Huang, 2002; Fu et al., 2007). In some plant tissues, PAs may act directly as antioxidant agents (Groppa et al., 2001). Foliar Spd application in creeping bentgrass has been shown to reduce lipid peroxidation and enhance drought tolerance; Li et al. (2015a) detected an up-regulation of peroxidase in 'Penn-A4' creeping bentgrass after exogenous Spd application. At the transcriptional level, we have found enriched transcripts (188/50) that are associated with antioxidants. Expression of a DE gene encoding a cationic peroxidase, SPC4, was up-regulated (2.2 fold) in response to Spd application under drought (WS vs DS). Peroxidase plays an important role in detoxifying H_2O_2 under stress and cationic peroxidase SPC4 is one of the peroxidase isoforms in sorghum grain (*Sorghum bicolor*; Dicko et al., 2006). Protein expression of SPC4 in a naturally drought tolerant purple feathergrass (*Stipa purpurea*) was higher than in the sensitive type, which indicates its importance in drought tolerance (Li et al.,

2016). Conversely, exogenously applied 1 mM Spd inhibited cell elongation and root growth of maize (Tisi et al., 2011), additional investigation of PA effects on the regulation of creeping bentgrass antioxidant systems is needed.

Enriched transcripts (46/23) were also found for other antioxidants. A gene encoding glutathione synthetase (GS) in Spd treated plants was up-regulated (8.9-fold) while down-regulation of this process was observed in drought (WC vs DC; -2.7-fold) and drought Spd treatment (WS vs DS; -9.9-fold). GS is one of the regulatory enzymes that catalyze formation of glutathione (GSH; Klapheck et al., 1992). GSH acts as substrate of glutathione S-transferase for antioxidant system to scavenge free radicals under stress or recycle ascorbic acid from its oxidized form to its reduced form by dehydroascorbate reductase (Szalai et al., 2009). In addition to the antioxidant effect of GSH, it induces production of H_2O_2 and Ca^{2+} for stomata closure in Arabidopsis (Munemasa et al., 2013). Thus, Spd may regulate transcripts involving in antioxidant production to fine tune the antioxidant system to improve drought tolerance.

Chemical priming, such as spraying PAs, is thought to involve epigenetic modification for a plant's stress memory to enable plants to better survive subsequent stresses (Peer et al., 2009; Minocha et al., 2014; Merewitz, 2016; Li and Liu, 2016). In this study, enriched transcripts (426/382) associated with chromatin organization (GO: 0006325) were identified. DE gene encoding histone deacetylase HDT3 was up-regulated for 8.7-fold. Although epigenetic modification occurs in histone or DNA through methylation, acetylation, demethylation, or deacetylation, histone deacetylation might be one of the mechanisms that promoted in response to applied Spd for drought tolerance. A better understanding of possible epigenetic changes for drought stress related to PAs in plants is still needed.

Processes associated with secondary metabolism can include important stress tolerance mechanisms. Gene encoding phosphoethanolamine N-methyltransferase 1 (PEAMT) for choline biosynthesis process was up-regulated by 8.3-fold due to drought while this process was down-regulated by 3.3-fold in the Spd treated plant (WC vs DS). PEAMT catalyzes the reaction by adding methyl groups for choline formation which serves as the precursor for biosynthesis of plasma membrane and glycine betaine. Enhanced choline and glycine betaine caused increased Arabidopsis osmotic stress tolerance (Zhang et al., 2010) and mutation of PEAMT showed early senescence and susceptibility to salinity stress (Mou et al., 2002). A gene encoding 1-deoxy-D-xylulose-5-phosphate synthase (DXS) was up regulated by 2.7 fold while it was down-regulated in Spd treated plants for 3.7 fold. DXS is the committed enzyme catalyzes the first step of isopentenyl diphosphate (IPP) formation within methylerythritol-4-phosphate pathway for terpene biosynthesis. In white grape, IPP derived compounds like phenylpropanoids, monoterpenes, and tocopherols were detected due to drought through transcriptome analysis (Savoi et al., 2016). Alkaloids, flavonoids (Larson, 1988), and anthocyanins (Noguees et al., 1998; Ramakrishna and Aswathanarayana, 2011) in pea (*Pisum sativum*) were induced by drought stress and were thought to act as non-enzymatic antioxidants. Therefore, Spd treated plants may not have been experiencing as much cellular stress damage. Spd treatment may have reduced the costs associated with the production of secondary metabolites that may be needed to reverse cellular stress damage incurred by creeping bentgrass under drought stress

Conclusions

A fully sequenced genome for creeping bentgrass is not yet available. RNA-Seq analysis coupled with functional annotation was successfully used to identify differentially expressed

genes due to drought stress and Spd treatment in creeping bentgrass. This study provides insight into gene transcripts and predicted functions in creeping bentgrass due to exogenous Spd application in response to drought. PA treatment primarily affected energy metabolism such as by transcripts associated with photosynthetic processes, triggered stress defenses such as antioxidant pathways, and other metabolic pathways under drought condition. As transcript levels can only suggest possible changes in protein expression, physiology, or biochemistry, future work is needed to more directly associate plant responses with the transcriptome changes found here due to PA and drought treatment. Specifically, PA regulation of phytohormones, carbon fixation processes, carbohydrate allocation and translocation, metabolomics, and proteomic studies may be beneficial to better understand PA effects on plants under stress.

Overall conclusions and future directions

The content of major PAs change in leaf tissue of creeping bentgrass in response to salt stress. Major differences in salt tolerance were not detected between two creeping bentgrass cultivars that were hypothesized to be differential in salt tolerance; thus, we cannot correlate any changes in PAs to tolerance or sensitivity of stress in creeping bentgrass. Specific changes in major PAs included shifts in Put and Spm content, which were two to five times higher in salt stressed leaf tissue compared with controls in early to the mid salt stress. Spd content did not show significant or consistent changes due to salt stress.

Since endogenous changes in PAs did occur, we have hypothesized that exogenously applied PAs could be a way to bolster stress tolerance, particularly Spd, which was not regulated naturally by salt stress. Additionally, PAs are known to be plant growth regulators. Products that could regulate turfgrass growth and boost plant stress tolerance are of major interest in the turfgrass management industry. Our results indicate that application of Spd 500 $\mu M \cdot L^{-1}$ promoted tillering rates of creeping bentgrass under optimal growth conditions in hydroponics. Under stress conditions, treatment with Spd (500 or 750 $\mu M \cdot L^{-1}$) or Spm (500 $\mu M \cdot L^{-1}$) enhanced creeping bentgrass photochemical efficiency, quantum yield of photosynthesis, leaf relative water content, and reduced electrolyte leakage, and lipid peroxidation compared to control plants.

The mechanisms associated with changes in PA content and how exogenous PA may regulate plant growth are not well understood. Therefore, to better understand these mechanisms we have performed transcriptome analysis using RNA-sequencing on leaf tissue that was either under drought stress or well-watered conditions that were either treated with

500 $\mu M \cdot L^{-1}$ Spd or untreated. Transcrimptome analysis has allowed us to evaluate differentially expressed genes due to drought and Spd application. About 22% and 19% of genes were either up- or down-regulated due to drought while 20% and 34% genes were either up- or down- regulated in response to Spd application, respectively. Gene ontology and enrichment analysis indicates that these DE genes were primarily associated with energy metabolism, transport, antioxidants, photosynthesis, signaling, and stress defense processes. For instance, higher expression level for genes encoding PSII polypeptide, sucrose synthase, peroxidase, and glutathione synthetase were detected in drought Spd treated plants compared with untreated drought stressed plants. This research is the first to provide transcriptome data for creeping bentgrass under drought stress.

Future work could include field evaluation of the efficacy of exogenously applied PA treatments, investigation of the cross-talk between PAs and other phytohormones that could play a role in the change in tillering or stress responses induced by PA treatment, investigation of the relationship between PA and photosynthesis, particularly the light reactions of photosynthesis, and a more detailed investigation into specific gene changes detected by transcriptomic analysis. Proteomic and metabolomics responses to PA treatments would also be important to corroborate transcriptome results found here. For example, PAs were shown to increase photochemical efficiency under drought and we found differential expression of PSII associated genes induced by PA. How PAs may specifically play a role in photochemical health and changes in proteins associated with light reaction centers may be warranted. Additionally, several transporters in the plant were alternately regulated by Spd treatment. As there is a lack of understanding of PA transport within plants, perhaps the genes

found here could be investigated more specifically for any possible function in transport of PAs.

APPENDIX

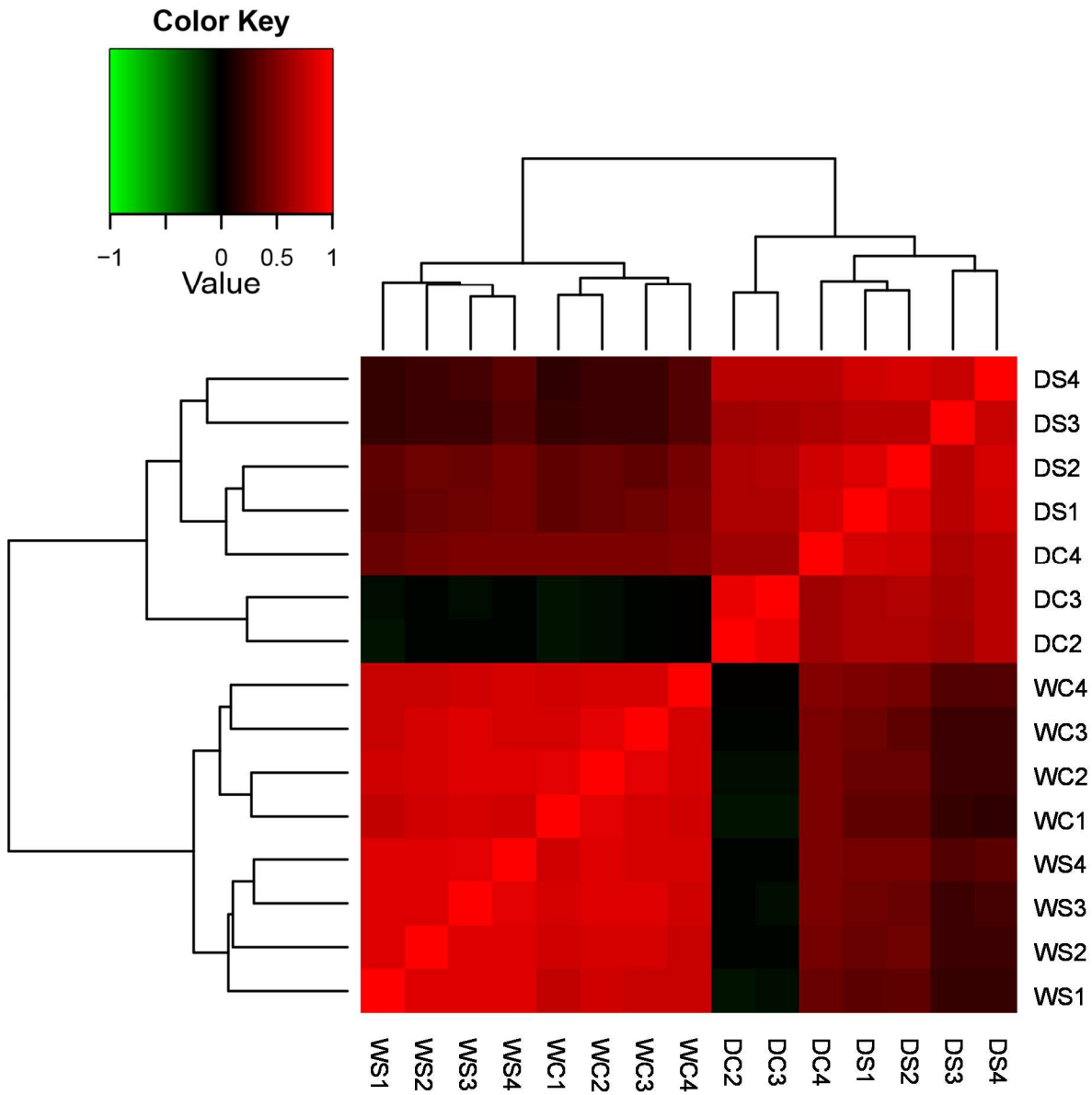

Figure 10: Pairwise analysis of correlation between biological replication for differentially expressed transcripts that used for de novo transcriptome assembly in creeping bentgrass exposed to the following experimental treatments. WS = watered plants treated with spermidine (Spd); WC = watered control plants (no Spd); DC = drought treated control plants (no Spd); DS = Drought plants treated with Spd. The dendrogram on the top was divided into two parts by representing water or drought treatment with all biological replications. The dendrogram on the left was separation of water or drought treatment with all biological replications.

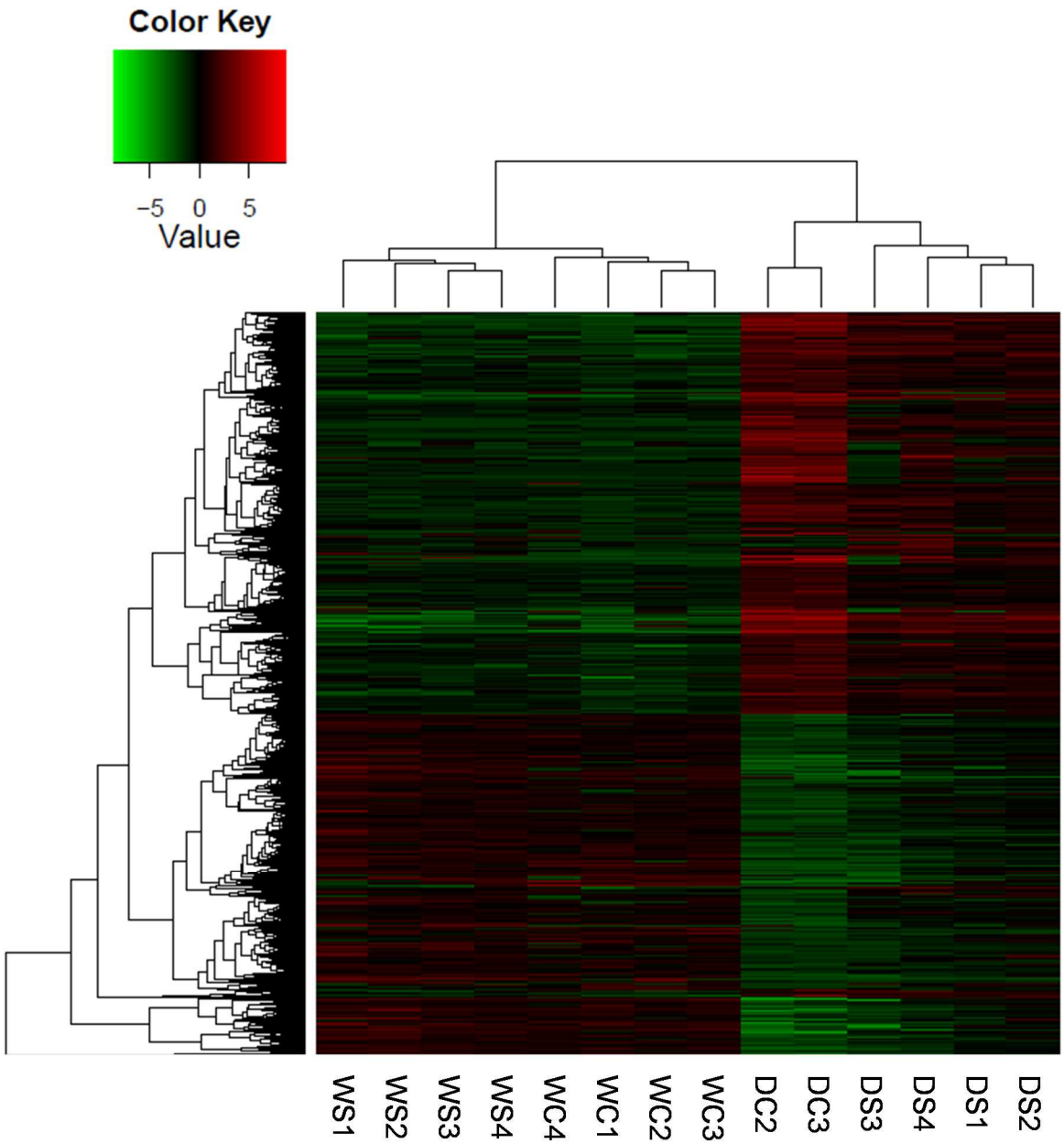

Figure 11: Heat map of all differentially expressed genes in creeping bentgrass for 14 samples with all biological replications exposed to the following experimental treatments. WS = watered plants treated with spermidine (Spd); WC = watered control plants (no Spd); DC = drought treated control plants (no Spd); DS = Drought plants treated with Spd. The dendrogram on the top was divided into two parts by representing water or drought treatment with all biological replications. The dendrogram on the left was separation of all transcripts involved in all possible biological processes. The processes that most relevant to drought and Spd were described in discussion part.

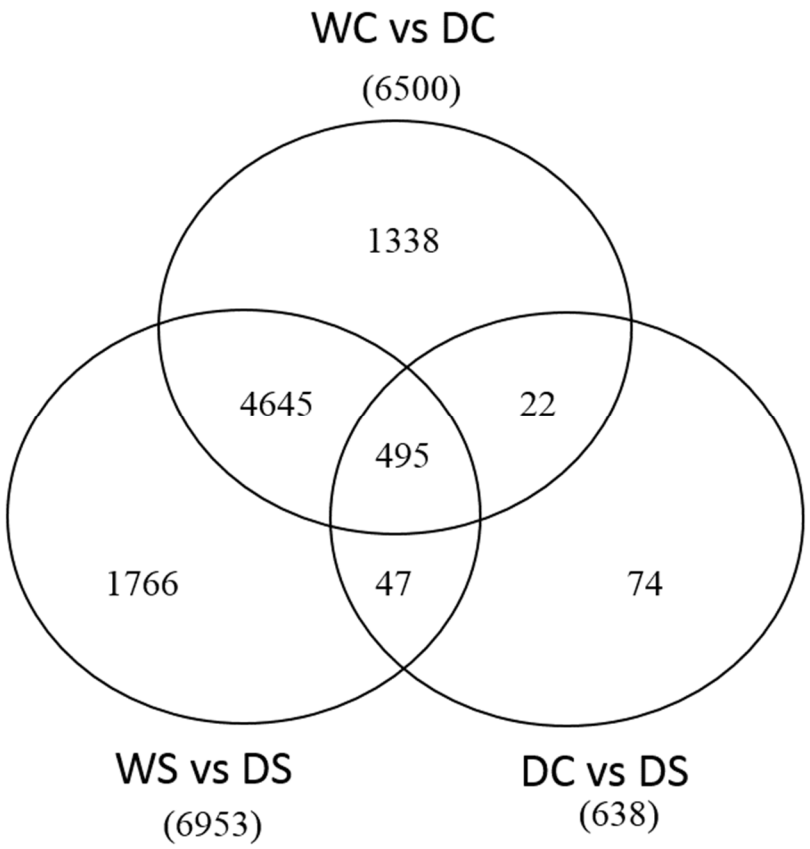

Figure 12: Venn diagram for all differentially expressed (DE) genes in creeping bentgrass exposed to the following experimental treatments. WS = watered plants treated with spermidine (Spd); WC = watered control plants (no Spd); DC = drought treated control plants (no Spd); DS = Drought plants treated with Spd. DE genes were quantified at a P value = 0.001 and a Log_2 fold change of > 2.0. Total DE genes for each comparison are shown in parenthesis.

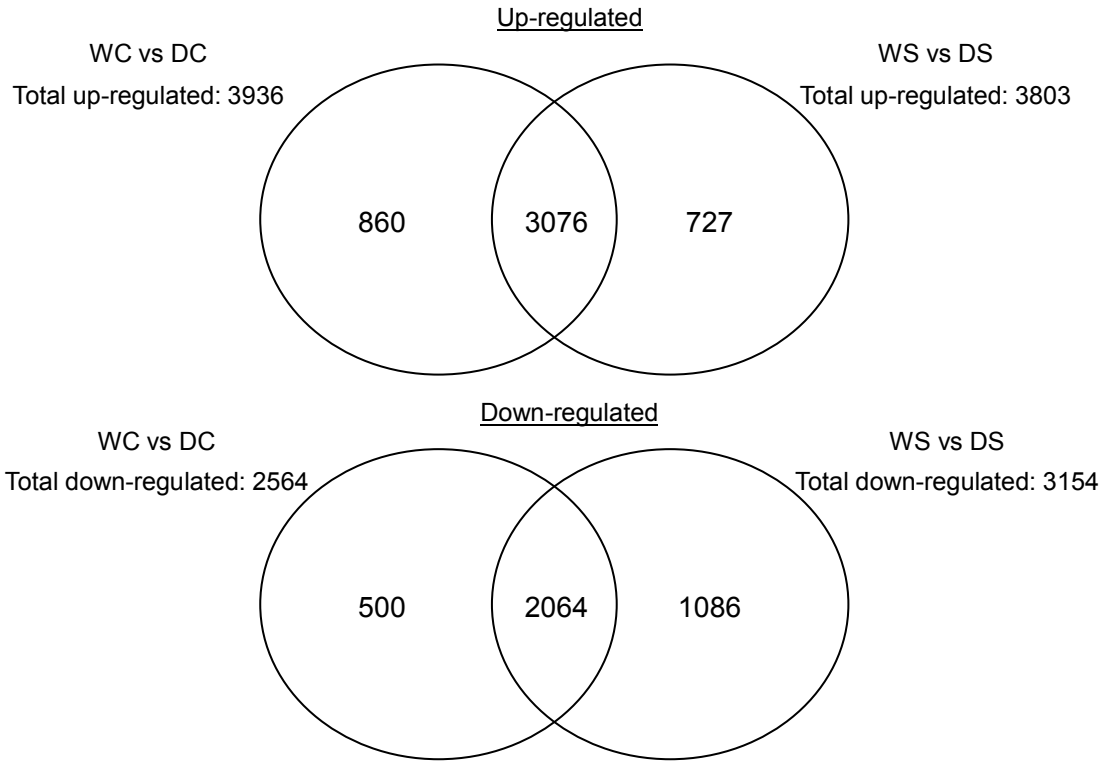

Figure 13: Venn diagram for all differentially expressed (DE) genes including those up-regulated and down-regulated in creeping bentgrass exposed to the following experimental treatments. WS = watered plants treated with spermidine (Spd); WC = watered control plants (no Spd); DC = drought treated control plants (no Spd); DS = Drought plants treated with Spd. DE genes were quantified at a P value = 0.001 and a Log_2 fold change of > 2.0. Total DE genes for each comparison are shown in parenthesis.

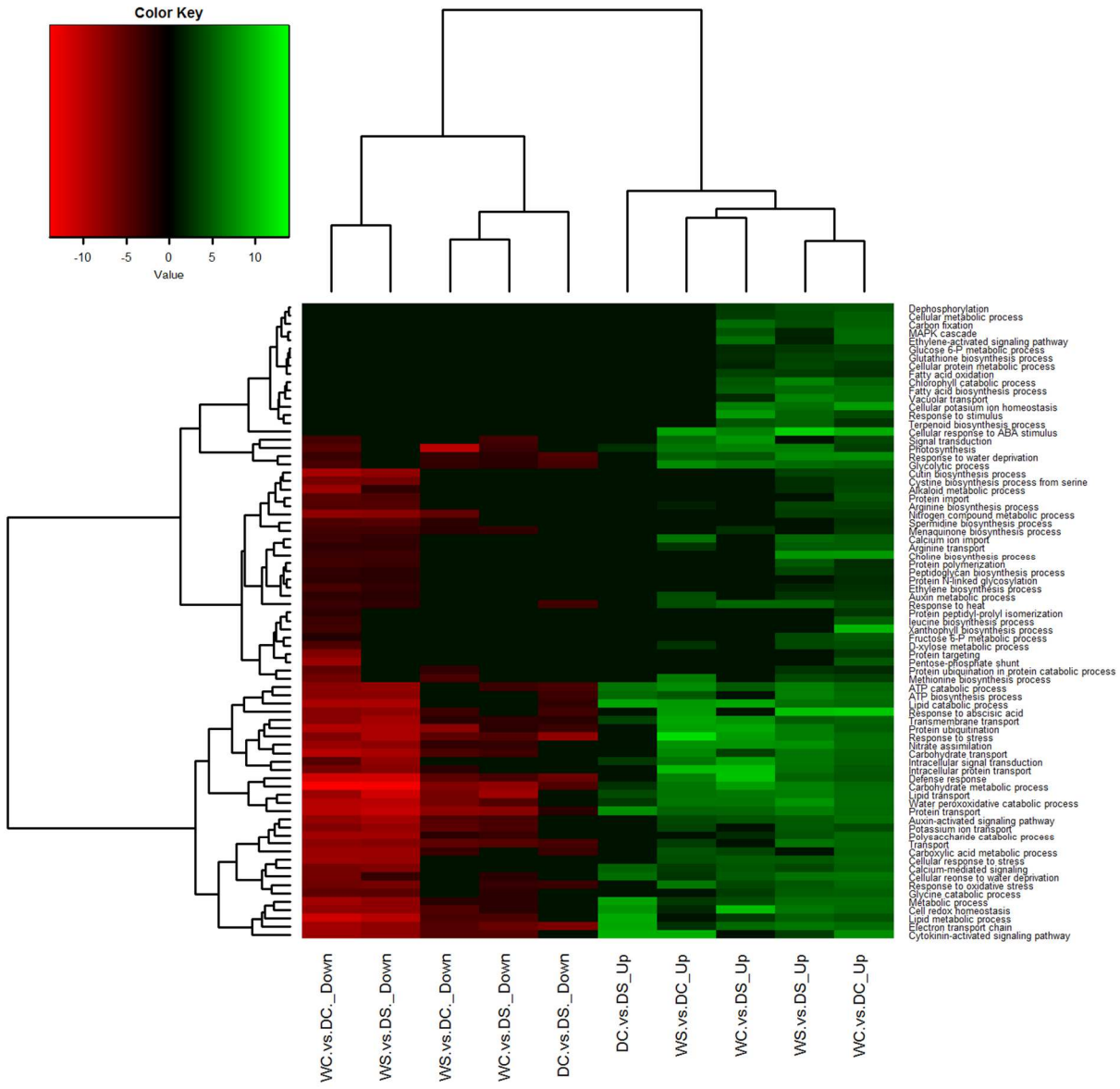

Figure 14: Heat map with cluster for differentially expressed (DE) genes at $\log_2$ scale in creeping bentgrass exposed to the following experimental treatments. WS = watered plants treated with spermidine (Spd); WC = watered control plants (no Spd); DC = drought treated control plants (no Spd); DS = Drought plants treated with Spd. The dendrogram on the top was divided into two parts by representing up- or down- regulation of the genes under each type of treatment comparisons. The dendrogram on the left was separation of different biological processes under different type of treatment comparisons. The bottom scale is pairs of different treatment comparisons. The legend is based on $\log_2$ fold changes of the genes and number of counts.

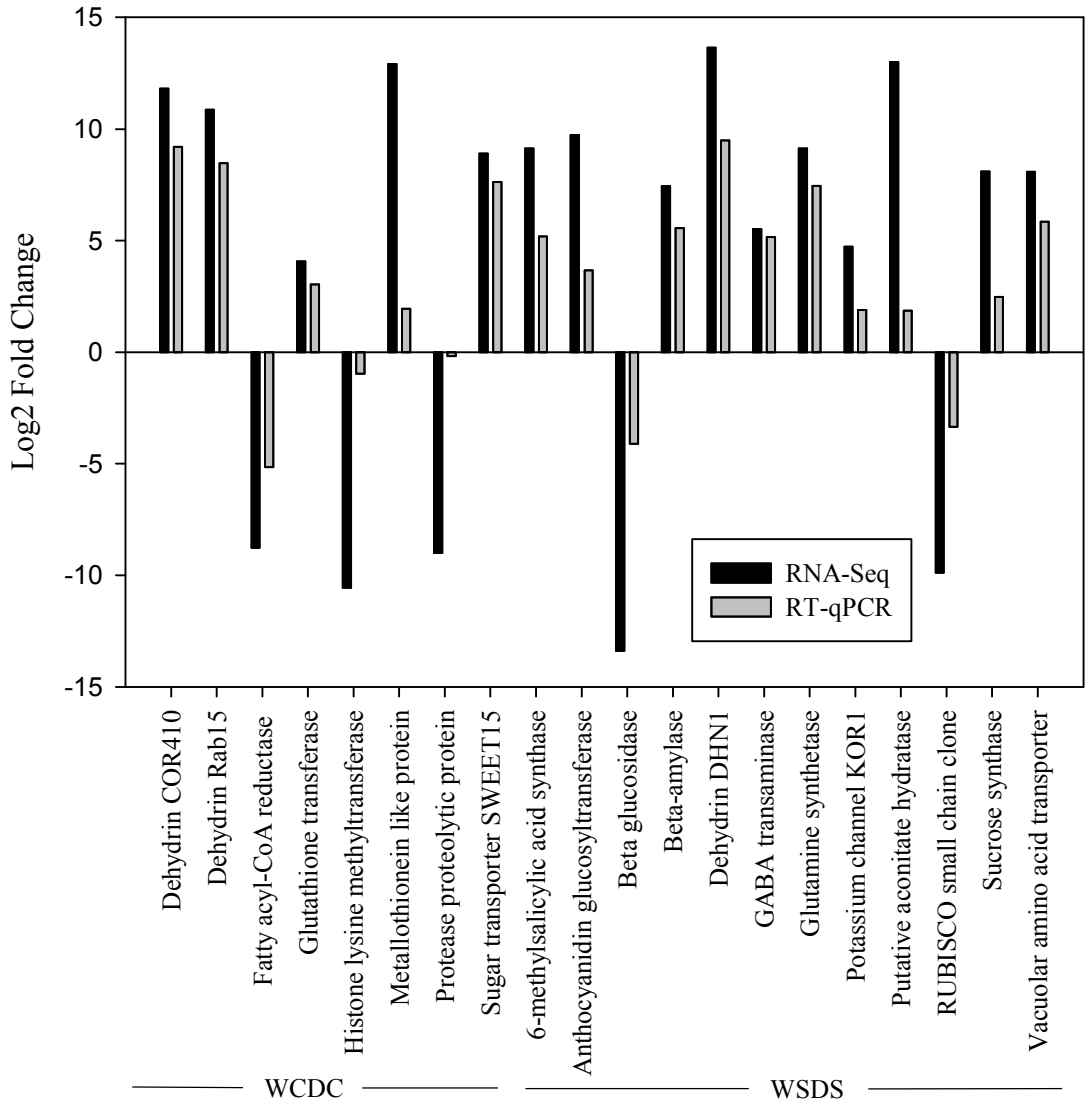

Figure 15: Log_2 fold changes of genes regulated by drought stress and/or spermidine (Spd) treatment based on RNA-Seq and qRT-PCR methods. Fold changes were calculated based on the following comparisons: WCDC, watered controls compared to drought controls, WSDS: watered plants treated with Spd compared to drought + Spd treatment.

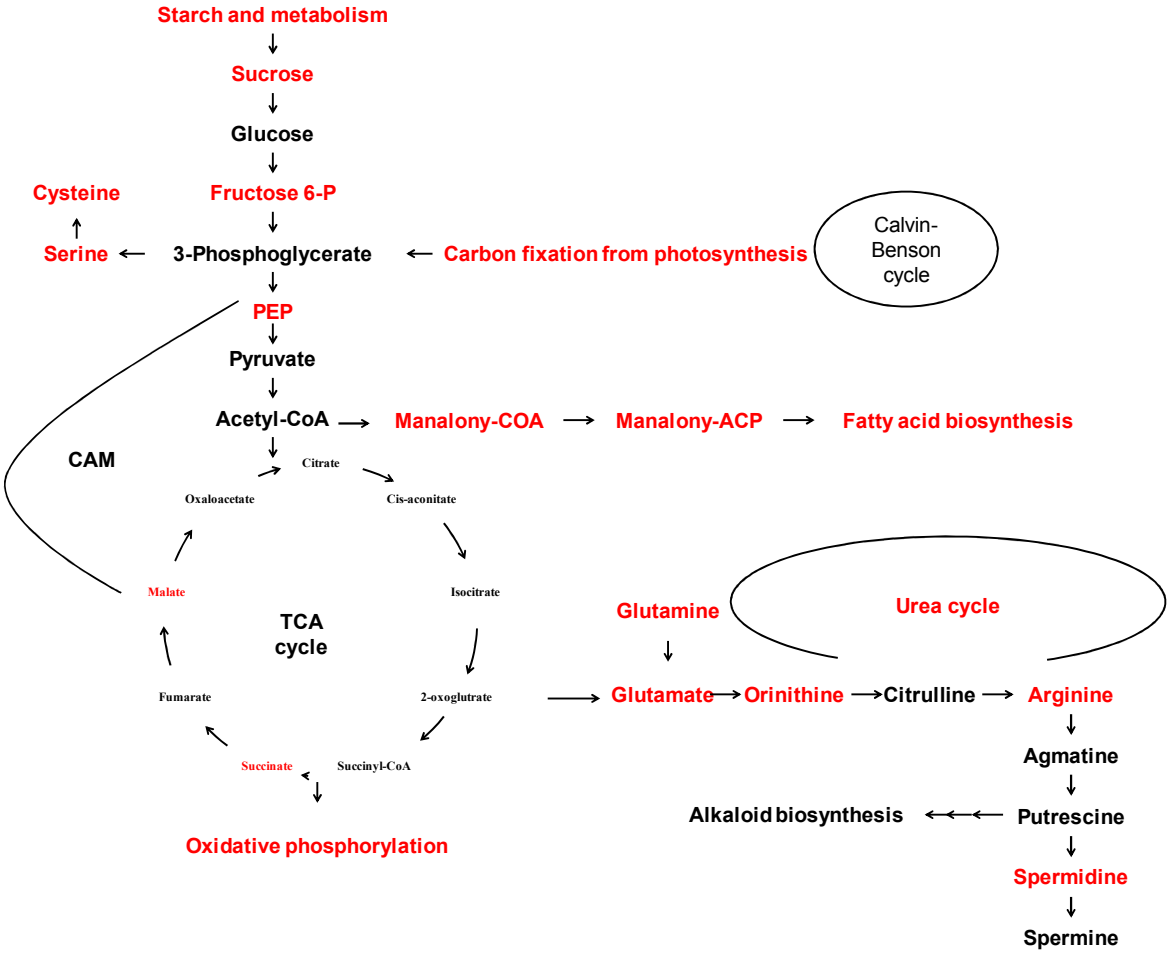

Figure 16: Proposed representation of TCA cycle intermediates, primary metabolites, and associated pathways. Red colored intermediates and pathways identified are for the transcripts that detected in kyoto encyclopedia of genes and genomes (KEGG) pathway to be enriched in response to Spd under drought in creeping bentgrass.

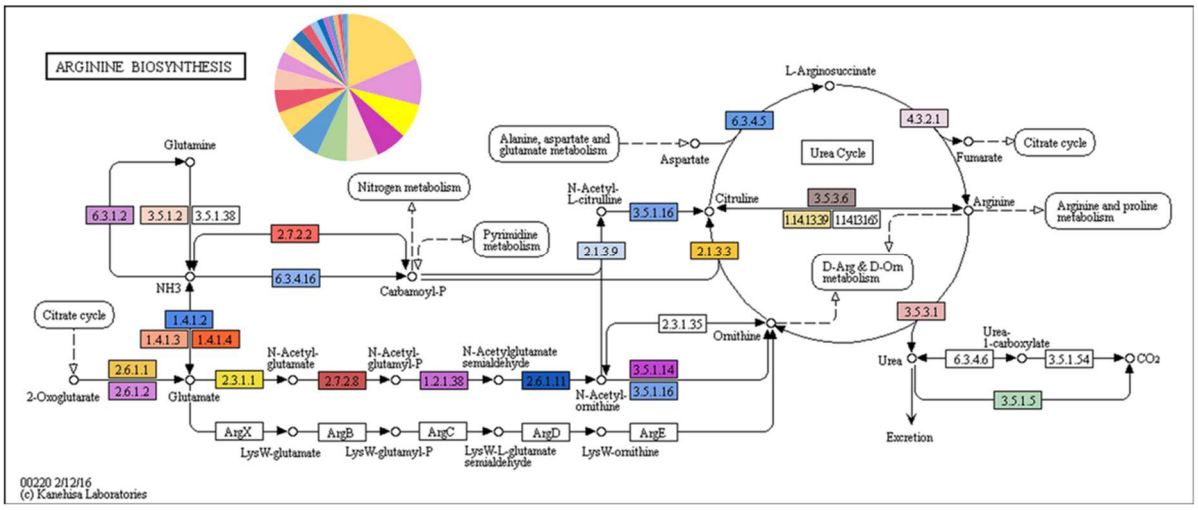

Figure 17: An example of the KEGG metabolic pathway map showing arginine biosynthesis. The color shaded boxes indicated the enzymes code (EC) number that is encoded by the transcripts detected in creeping bentgrass and in the genome of the KEGG pathway maps by comparing this enzyme coding gene sequence with the reference sequences. Circle is a metabolic compound in the KEGG pathway map. Number of sequences detected for enzymes with color shaded boxes was presented in the pie chart.

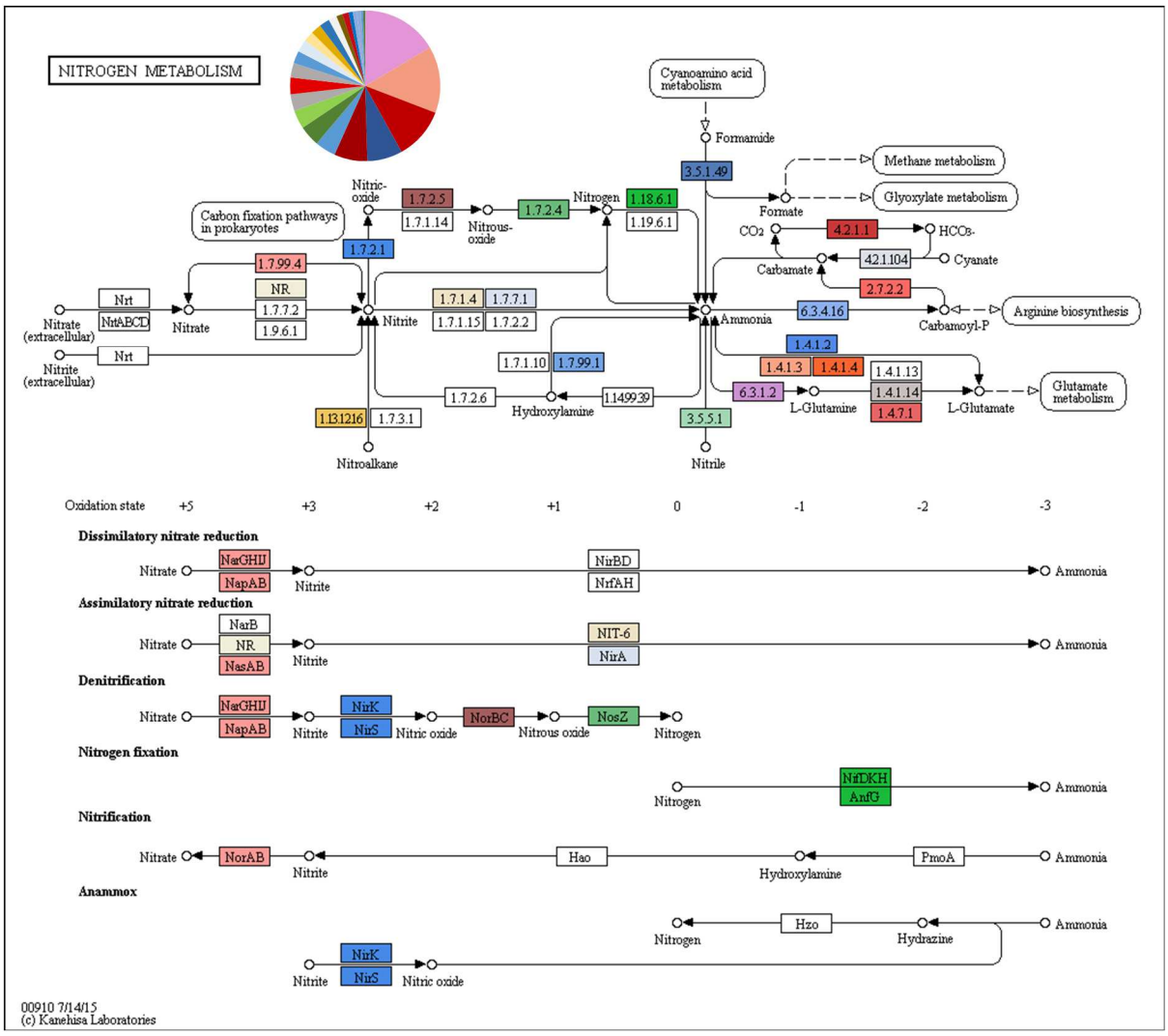

Figure 18: This is an example of the KEGG metabolic pathway map showing nitrogen metabolism. The color shaded boxes indicated the enzymes code (EC) number that is encoded by the transcripts detected in creeping bentgrass and in the genome of the KEGG pathway maps by comparing this enzyme coding gene sequence with the reference sequences. Circle is a metabolic compound in the KEGG pathway map. Number of sequences detected for enzymes with color shaded boxes was presented in the pie chart.

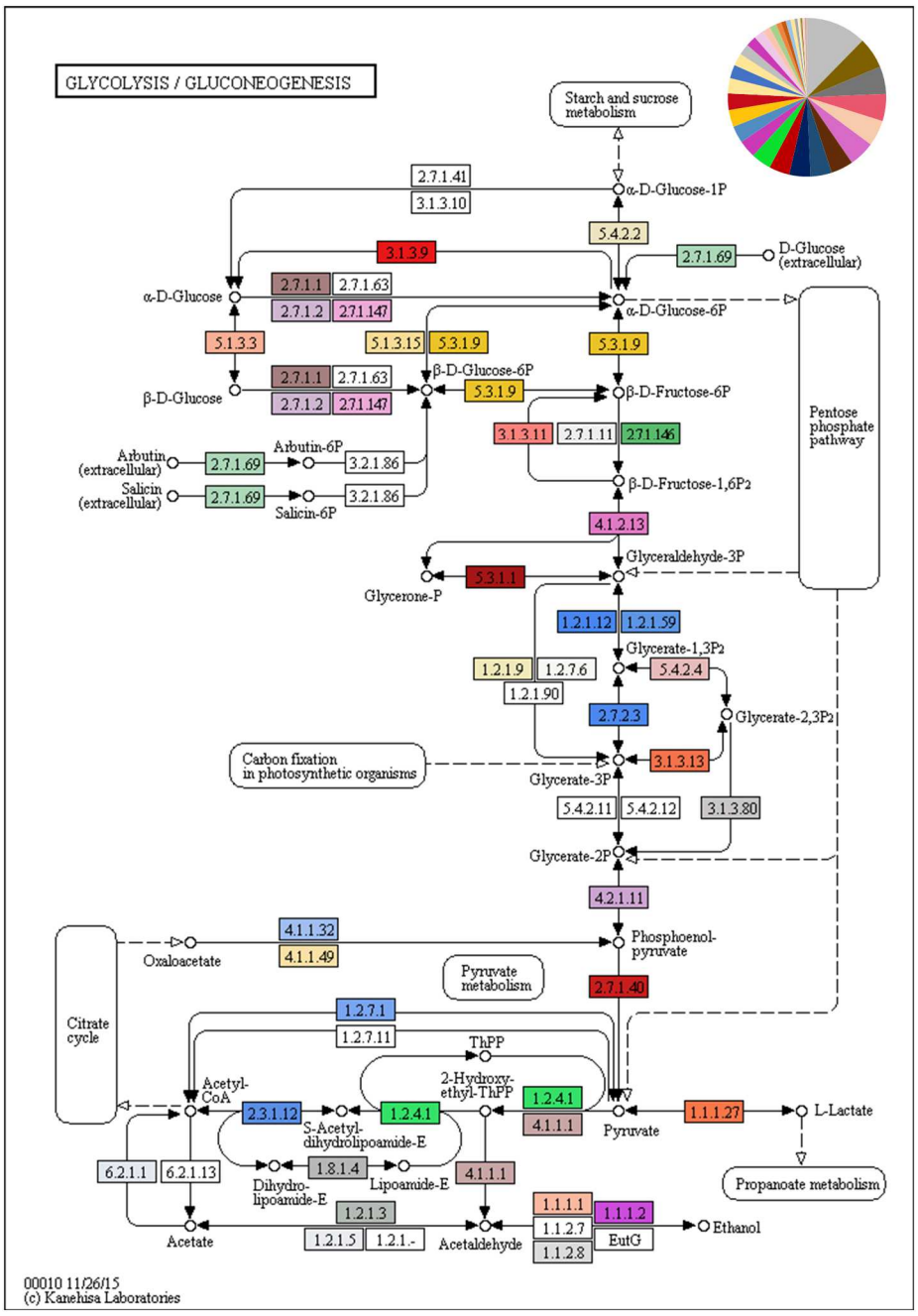

Figure 19: This is an example of the KEGG metabolic pathway map showing glycolysis pathway. The color shaded boxes indicated the enzymes code (EC) number that is encoded by the transcripts detected in creeping bentgrass and in the genome of the KEGG pathway maps by comparing this enzyme coding gene sequence with the reference sequences. Circle is a metabolic compound in the KEGG pathway map. Number of sequences detected for enzymes with color shaded boxes was presented in the pie chart.

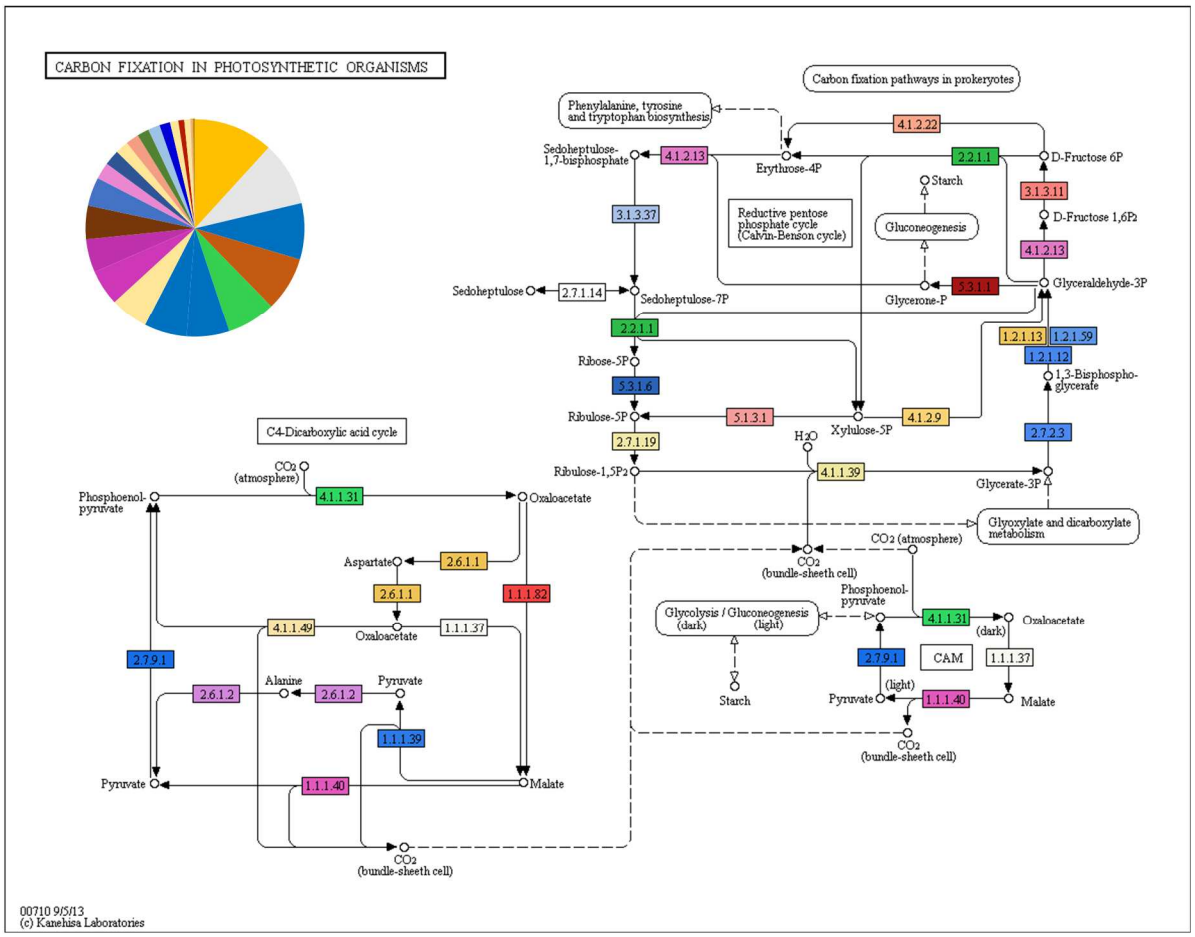

Figure 20: This is an example of the KEGG metabolic pathway map showing carbon fixation in photosynthesis pathway. The color shaded boxes indicated the enzymes code (EC) number that is encoded by the transcripts detected in creeping bentgrass and in the genome of the KEGG pathway maps by comparing this enzyme coding gene sequence with the reference sequences. Circle is a metabolic compound in the KEGG pathway map. Number of sequences detected for enzymes with color shaded boxes was presented in the pie chart.

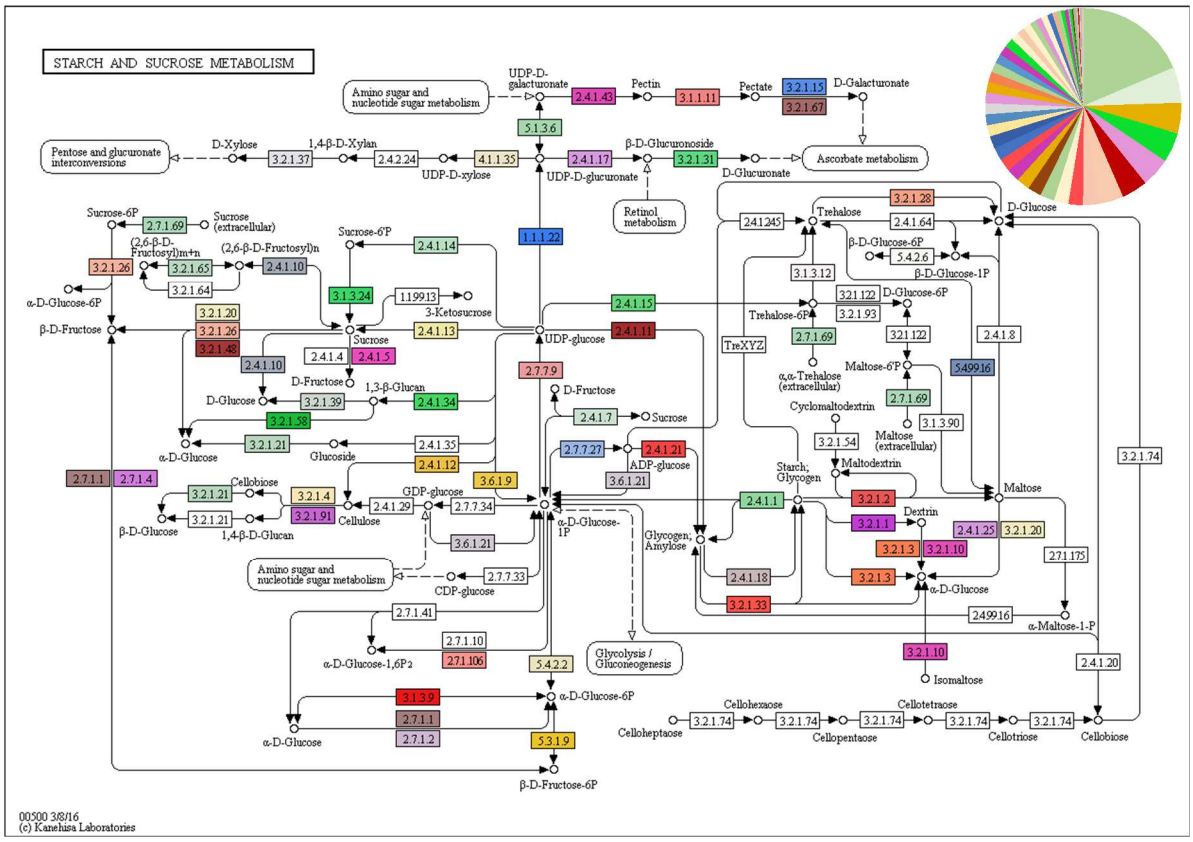

Figure 21: This is an example of the KEGG metabolic pathway map showing starch and sucrose metabolism. The color shaded boxes indicated the enzymes code (EC) number that is encoded by the transcripts detected in creeping bentgrass and in the genome of the KEGG pathway maps by comparing this enzyme coding gene sequence with the reference sequences. Circle is a metabolic compound in the KEGG pathway map. Number of sequences detected for enzymes with color shaded boxes was presented in the pie chart.

Table 5: Trimming summary of read data after processing through Trimmomatic to remove adapters and low quality bases.

Sample ID	Lane	Treatment 1	Treatment 2	PF read pairs	Trimmed read pairs	% Retained
DC2	1	Drought	No Spd	7,633,388.00	6,768,936.00	88.68%
DC2	2	Drought	No Spd	7,463,655.00	6,667,913.00	89.34%
DC2 total				**15,097,043.00**	**13,436,849.00**	**89.00%**
DC3	1	Drought	No Spd	6,691,115.00	5,951,842.00	88.95%
DC3	2	Drought	No Spd	6,539,554.00	5,864,381.00	89.68%
DC3 total				**13,230,669.00**	**11,816,223.00**	**89.31%**
DC4	1	Drought	No Spd	9,439,129.00	8,477,791.00	89.82%
DC4	2	Drought	No Spd	9,244,949.00	8,360,962.00	90.44%
DC4 total				**18,684,078.00**	**16,838,753.00**	**90.12%**
DS1	1	Drought	Spd	8,872,679.00	7,917,624.00	89.24%
DS1	2	Drought	Spd	8,658,861.00	7,786,442.00	89.92%
DS1 total				**17,531,540.00**	**15,704,066.00**	**89.58%**
DS3	1	Drought	Spd	8,474,556.00	7,554,984.00	89.15%
DS3	2	Drought	Spd	8,296,154.00	7,453,665.00	89.84%
DS3 total				**16,770,710.00**	**15,008,649.00**	**89.49%**
DS2	1	Drought	Spd	9,115,771.00	8,078,536.00	88.62%
DS2	2	Drought	Spd	8,932,207.00	7,982,230.00	89.36%
DS2 total				**18,047,978.00**	**16,060,766.00**	**88.99%**
DS4	1	Drought	Spd	14,110,739.00	12,447,602.00	88.21%
DS4	2	Drought	Spd	13,832,121.00	12,291,452.00	88.86%
DS4 total				**27,942,860.00**	**24,739,054.00**	**88.53%**
WC1	1	Water	No Spd	14,963,543.00	13,461,288.00	89.96%
WC1	2	Water	No Spd	14,680,775.00	13,307,337.00	90.64%
WC1 total				**29,644,318.00**	**26,768,625.00**	**90.30%**
WC3	1	Water	No Spd	7,047,447.00	6,380,929.00	90.54%
WC3	2	Water	No Spd	6,855,985.00	6,250,295.00	91.17%
WC3 total				**13,903,432.00**	**12,631,224.00**	**90.85%**

Table 5 (cont'd)

WC2	1	Water	No Spd	7,530,771.00	6,734,579.00	89.43%
WC2	2	Water	No Spd	7,362,579.00	6,635,802.00	90.13%
WC2 total				**14,893,350.00**	**13,370,381.00**	**89.77%**
WC4	1	Water	No Spd	6,961,569.00	6,202,189.00	89.09%
WC4	2	Water	No Spd	6,819,730.00	6,125,395.00	89.82%
WC4 total				**13,781,299.00**	**12,327,584.00**	**89.45%**
WS2	1	Water	Spd	6,788,605.00	5,924,992.00	87.28%
WS2	2	Water	Spd	6,642,392.00	5,846,594.00	88.02%
WS2 total				**13,430,997.00**	**11,771,586.00**	**87.64%**
WS1	1	Water	Spd	9,229,747.00	8,024,340.00	86.94%
WS1	2	Water	Spd	9,061,114.00	7,954,178.00	87.78%
WS1 total				**18,290,861.00**	**15,978,518.00**	**87.36%**
WS3	1	Water	Spd	7,706,301.00	6,894,431.00	89.46%
WS3	2	Water	Spd	7,572,250.00	6,823,541.00	90.11%
WS3 total				**15,278,551.00**	**13,717,972.00**	**89.79%**
WS4	1	Water	Spd	8,011,523.00	7,164,022.00	89.42%
WS4	2	Water	Spd	7,830,795.00	7,055,629.00	90.10%
WS4 total				**15,842,318.00**	**14,219,651.00**	**89.76%**
Grand total				**262,370,004.00**	**234,389,901.00**	**89.34%**

PF= Passed filter measured by illumina sequencer.

Table 6: Primer pairs used for quantitative real-time-PCR analysis to confirm RNA-Seq results from creeping bentgrass.

Gene ID	Gene name	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
c215662_g1_i2	Putative aconitate hydratase	CTCACTCGGCCTTACTGGACAT	CTCGCTGACGTCGGTAGGAA
Actin	Actin	TCCAGCAAGGTCAAGACGAA	GCCATACTGTGCCAATCTATGAAG
c207964_g1_i6	Gamma-aminobutyrate transaminase 1	AGCATATCACGGATCAACATTGA	TCTGGTGCAGGGCAGGAA
c210911_g1_i5	Beta-amylase 1	GGCCAGCAACGGACAACT	GATCCGCTAGTGTAGGGAACGA
c206464_g1_i1	Potassium channel KOR1	CGCAGTCCTCTCCACATAGCT	ACCAGCATCTTCGCCATCA
c212560_g1_i7	ATP-dependent Clp protease proteolytic subunit-related protein 3	TTACACTGAGCACAGGCCTAGAAG	ACTATTCATCCAATCAAGGTACATCAA
c211457_g3_i7	Dehydrin Rab15	GACCGACCGAGCCACACT	TCGCGCTGACCTTGGAA
c201145_g2_i4	Ribulose bisphosphate carboxylase small chain clone 512	GACTCTGCTCCAGGGTTTGTT	GTCCTTATGTGTAGCCGGTTGT
c199947_g1_i4	6-methylsalicylic acid synthase	GTACAGAGGATCTTGGCGAGCTA	AGCTCCATCAACATCTCCACAA
c211568_g1_i7	Histone-lysine N-methyltransferase setd3	TGCTGCATGGCTGTCTTCAG	CAAGGCGACACGGTATGGT
c201596_g3_i1	Sucrose synthase 4	CCGTGAGCTGGTGAAGAGTGT	GGCACTGACCACGGTTAGTGT
c217665_g1_i3	Bidirectional sugar transporter SWEET15	GAAGAAGCCATCCGTGACCA	CTGCCAAACCCAAGCGAATC
c219508_g1_i4	Vacuolar amino acid transporter 1	GCCAGTGGCCGCAGAGT	GGTGACGCAGCGATTGTGTA

Table 6 (cont'd)

c205816_g1_i2	Anthocyanidin 5 3-O-glucosyltransferase	GACATGTCGACCACGATGCT	TGTACTGACGCAACCGTTCCT
c192083_g2_i5	Dehydrin COR410	CCAGGCCAGCACTGAGCTA	ACTGGCACCTCAACCTTCACA
c210664_g1_i5	Dehydrin DHN1	TGCACATGTAATACGCACTATCTCA	GACGCGATAGCTAGGGAACATAC
c216031_g2_i11	Fatty acyl-CoA reductase 1	CGGTTGCACAGTTGACATTGT	GGCGAAGCATCTCAATTGGA
c214281_g1_i7	Beta-glucosidase 5	GGAGGATGGCTAAGCCCTACA	GGAAGCACACATCTGCAAATTC
c221086_g1_i3	Glutamine synthetase cytosolic isozyme 1-3	ACATCCTGGTTATCTGTGACTGCTAT	CCGCTTGTTGGACGGAAT
c211186_g2_i6	Glutathione transferase GST 23	CAGTAGCACGAAGCACTCTGTCTT	CACATGCCGAACACCTTCAC
c198465_g2_i4	Dehydrin Rab15	GCGTAATCAAGCATGCTCTGAAT	GGAACGTGGACGATGAGGAA

Table 7: The number of filtered and trimmed read pairs (each pair representing one library fragment) from each sample used for de novo transcriptome assembly, number of alignments, and corresponding alignment efficiency value as percent of aligned reads (% Aligned).

Sample ID	Fragments (Read Pairs) in Assembly	Number Aligned*	% Aligned
DC2	13,436,849	7,836,081	58.3%
DC3	11,816,223	6,906,540	58.4%
DS1	15,704,066	9,553,299	60.8%
DS3	15,008,649	11,181,811	74.5%
DS2	16,060,766	9,743,475	60.7%
DS4	24,739,054	14,837,478	60.0%
WC1	26,768,625	16,079,327	60.1%
WC2	12,631,224	7,287,334	57.7%
WC3	13,370,381	7,914,263	59.2%
WC4	12,327,584	7,334,851	59.5%
WS2	11,771,586	7,052,781	59.9%
WS1	15,978,518	9,474,455	59.3%
WS3	13,717,972	8,084,610	58.9%
WS4	14,219,651	8,410,619	59.1%
Grand	234,389,901	131,696,924	56.2%

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