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RESEARCH PAPER

Protein accumulation in leaves and roots associated with improved drought tolerance in creeping bentgrass expressing an *ipt* gene for cytokinin synthesis

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Abstract

Cytokinins (CKs) may be involved in the regulation of plant adaptation to drought stress. The objectives of the study were to identify proteomic changes in leaves and roots in relation to improved drought tolerance in transgenic creeping bentgrass (*Agrostis stolonifera*) containing a senescence-activated promoter (*SAG12*) and the isopentyl transferase (*ipt*) transgene that increases endogenous CK content. Leaves of *SAG12-ipt* bentgrass exhibited less severe senescence under water stress, as demonstrated by maintaining lower electrolyte leakage and lipid peroxidation, and higher photochemical efficiency (F_v/F_m), compared with the null transformant (NT) plants. *SAG12-ipt* plants had higher root/shoot ratios and lower lipid peroxidation in leaves under water stress than the NT plants. The suppression of drought-induced leaf senescence and root dieback in the transgenic plants was associated with the maintenance of greater antioxidant enzyme activities (superoxide dismutase, peroxidase, and catalase). The *SAG12-ipt* and NT plants exhibited differential protein expression patterns under well-watered and drought conditions in both leaves and roots. Under equivalent leaf water deficit (47% relative water content), *SAG12-ipt* plants maintained higher abundance of proteins involved in (i) energy production within both photosynthesis and respiration [ribulose 1,5-bisphosphate carboxylase (RuBisCO) and glyceraldehyde phosphate dehydrogenase (GAPDH)]; (ii) amino acid synthesis (methionine and glutamine); (iii) protein synthesis and destination [chloroplastic elongation factor (EF-Tu) and protein disulphide isomerases (PDIs)]; and (iv) antioxidant defence system (catalase and peroxidase) than the NT plants. These results suggest that increased endogenous CKs under drought stress may directly or indirectly regulate protein abundance and enzymatic activities involved in the above-mentioned metabolic processes, thereby enhancing plant drought tolerance.

Key words: Cytokinins, drought stress, isopentyl transferase, perennial grass, proteome, senescence, senescence-activated promoter, turfgrass.

Introduction

Exposure of plants to water stress causes many physiological changes within plant cells, including hormonal metabolism and proteomic changes (Salekdeh *et al.*, 2002*a*, *b*; Davies, 2010). Endogenous cytokinin (CK) biosynthesis, content, translocation, and activity decline in response to water stress (Yang *et al.*, 2002; Kudoyarova *et al.*, 2006). Maintenance of CKs, whether through increasing biosynthesis, reducing CK degradation, or increasing CK stability

Abbreviations: ABA, abscisic acid; CA, carbonic anhydrase; CAT, catalase; CK, cytokinin; CP, 2-Cys peroxiredoxin; EL, electrolyte leakage; F_v/F_m, photochemical efficiency; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, glutamine synthetase; HSP, heat-shock protein; IDH, isocitrate dehydrogenase; IEF, isoelectric focusing; iPa, isopentenyl adenine; ipt, adenine isopentenyl transferase gene; MALDI, matrix-assisted laser desorption/ionization; MDA, malondialdehyde; MS, methionine synthase; NT, null transformant; OA, osmotic adjustment; OEE, oxygen-evolving complex; PDI, protein disulphide isomerase; 6PGDH, 6-phosphogluconate dehydrogenase; POD, peroxidase; PSAK, photosystem I subunit K; RWC, relative water content; RuBisCO, ribulose-1,5-bisphosphate carboxylase oxygenase; SAG12, senescence-activated promoter 12; SAMS, S-adenosylmethionine synthetase; SOD, superoxide dismutase; SWC, soil water content; TDR, time domain reflectometry; TOF, time of flight; TPI, triose phosphate isomerase; WUE, water use efficiency; ZR, zeatin riboside.

during stress conditions, has been found to be an important factor regulating plant responses to environmental stress. This has been supported by research using transgenic modification of the CK content in various plant species, such as *Arabidopsis* (*Arabidopsis thaliana*) (Medford *et al.*, 1989; Zhang *et al.*, 2000), lettuce (*Lactuca sativa*) (McCabe *et al.*, 2001), tobacco (*Nicotiana tabacum*) (Rivero *et al.*, 2007, 2009), petunia (*Petunia×hybrida*) (Clark *et al.* 2004), tall fescue (*Festuca arundinacea*) (Hu *et al.*, 2005), and creeping bentgrass (*Agrostis stolonifera*) (Xu *et al.*, 2009; Merewitz *et al.*, 2010, 2011).

Transgenic modification of plants to incorporate the *ipt* gene encoding an enzyme in the CK biosynthesis pathway, adenine isopentenyl transferase, increases endogenous CK content, resulting in improved drought tolerance in various plant species (Clark *et al.* 2004; Rivero *et al.*, 2007; Merewitz *et al.*, 2010, 2011; P Zhang *et al.*, 2010). Merewitz *et al.* (2010, 2011) reported that compared with null transformant (NT) control plants, creeping bentgrass (a C_3 perennial grass species) containing the *ipt* gene under a senescence-activated promoter (*SAG12-ipt*) exhibited higher photosynthesis rates, photochemical efficiency (F_v/F_m), leaf chlorophyll content, osmotic adjustment, and water use efficiency (WUE), as well as enhanced root growth, and root viability under drought stress. Rivero *et al.* (2007) found that *ipt* transgenic tobacco had improved drought tolerance, which was manifested by maintaining a higher water content and photosynthetic activity, and displayed minimal yield loss during drought. They attributed the improved drought tolerance in *SAG12-ipt* transgenic tobacco to the up-regulation of photorespiration protection of photosynthesis under drought stress (Rivero *et al.*, 2009). Clark *et al.* (2004) found that *ipt* transgenic lines of petunia exhibited delayed leaf senescence and increased the number of branches, but decreased adventitious rooting. Transgenic cassava (*Manihot esculenta*) plants with *ipt* maintained higher chlorophyll content and an early storage root bulking in comparison with wild-type plants (P Zhang *et al.*, 2010).

Despite knowledge of CK-mediated drought responses in some monocot species and many dicot species, how *ipt* gene-regulated CK synthesis during drought stress regulates metabolic processes, such as photosynthesis, antioxidant metabolism, osmotic adjustment, and other physiological characteristics underlying drought tolerance, is not well understood. It is commonly known that hormonal and proteomic changes are tightly linked and may coordinately regulate plant responses to drought for stress perception, signalling, and metabolic regulation (Bray, 1997). Questions still remain regarding what specific protein changes may occur in leaves and roots of creeping bentgrass with elevated CK content that has been found to promote drought tolerance. Two-dimensional PAGE has been widely used to differentiate proteomic responses between drought-tolerant and drought-sensitive plants (Riccardi *et al.*, 1998; C Xu *et al.*, 2008, 2010; Y Xu *et al.*, 2009; Xu and Huang, 2010; Zhao *et al.*, 2011) and has allowed for the successful identification of proteins regulating the plant defence response or cellular damage caused by drought (Riccardi *et al.*, 1998). Identification of specific changes in enzymatic activities and abundance of proteins due to elevated CK content may aid in elucidating the relationship of CKs to various drought protection responses.

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

Materials and methods

Plant material and growth conditions

Transgenic creeping bentgrass plants were produced by the *Agrobacterium* transformation method as described previously (Xu *et al.*, 2009; Merewitz *et al.*, 2010, 2011; Xing *et al.*, 2010). Plant material included a null transformed line of 'Penncross' (NT) and a *SAG12-ipt* transgenic line (S41). The *SAG12* promoter is expressed in an autoregulated manner under stress conditions to prevent excess CKs from accumulating. *SAG12-ipt* expression caused greater levels of CKs and better drought tolerance than in NT plants in previous drought studies (Merewitz *et al.*, 2010, 2011). Transgene expression determined by northern blot analysis and changes in hormone content of *SAG12-ipt* lines under drought stress relative to NT have been previously reported (Merewitz *et al.*, 2010). All plant material was vegetatively propagated in a greenhouse in January 2009 and transplanted into PVC tubes (40 cm in height×10.16 cm in diameter) containing an equal volume of 1:1 fine sand:soil mix (fine-loamy, mixed mesic Typic Hapludult type soil). Greenhouse conditions were controlled to maintain natural light and supplemental sodium lamps when necessary at ~600 μmol m^{-2} s^{-1} photosynthetic photon flux density at canopy height for a 12 h photoperiod and an average air temperature of 21 °C/14 °C (day/night). Plants were watered daily and fertilized once per week with Hoagland's nutrient solution (Hoagland and Arnon, 1950). Plants were allowed to establish fully in grass canopy and root systems during December–February 2009 for ~60 d in the greenhouse. Plants were then transferred to a controlled-environment growth chamber in February 2009 (Conviron, Winnipeg, Canada) where they were acclimated to the growth chamber conditions for 10 d. The chamber was set to maintain 20/15 °C (day/night) temperatures, 12 h photoperiod, 60% relative humidity, and 500 μmol m^{-2} s^{-1} photosynthetic photon flux density at canopy height. Watering treatments were imposed in the growth chamber on 3 March 2009.

Water stress treatments

Water treatments consisted of a well-watered control or water stress by withholding irrigation for both NT and *SAG12-ipt* plants (40 plants of each). Soil volumetric water content (SWC) was determined with the time domain reflectometry (TDR) method (Topp *et al.*, 1980) using a Trase TDR instrument (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). SWC was measured with three-pronged waveguide probes (20 cm in length, spaced 2.54 cm apart) installed vertically in each pot, four probes in the control treatment and four probes in the water stress treatment (four replicates in each line). Pot capacity of the soil water was ~25%.

Physiological evaluation

All physiological measurements and protein sampling were carried out on four replicated pots when the SWC reached an average of

22, 18, 15, 10, or 5% in pots of both plant lines, which occurred over a period of 14 d of water stress treatment. This was done so that comparisons within the physiological attributes and protein responses between lines can be made at a given soil moisture level or at the same water deficit level.

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

Leaf electrolyte leakage (EL) measurement was performed to estimate cell membrane stability and indicate drought damage severity. Leaf samples of ~10 leaves were taken from each plant, washed in de-ionized water four times, immersed in 25–30 ml of de-ionized water, and placed on the shaker for 24 h. The conductivity of the immersion water containing the living leaf tissue was measured as initial conductivity (C_i). The samples were then autoclaved, placed on the shaker for 24 h, and the conductivity of the resulting water containing the dead tissue was measured as maximum conductivity (C_{max}). The percentage EL was calculated as $C_i/C_{max}\times100$ (Blum, 1981).

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

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

Antioxidant activity and malondialdehyde (MDA) content

Activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), and MDA content were determined based on the protocols described in Xu and Huang (2004). Briefly, a fresh leaf or root sample of ~0.5–1.0 g was collected from each plant, frozen immediately in liquid nitrogen, and stored at –80 °C until use. For enzymes and MDA extraction, frozen samples were homogenized with 7 ml of 50 mM phosphate buffer solution (pH 7.0), ground in a mortar on ice, and centrifuged at 20 000 *g* for 25 min at 4 °C. The supernatant was used to evaluate total soluble protein, enzyme activity, and MDA content. Protein content was based on comparison with bovine serum albumin (BSA) as a standard (Bradford, 1976). SOD activity was measured according to the method of Zhang and Kirkham (1996) and Xu and Huang (2004). One unit of SOD activity was defined as the amount of SOD required to cause 50% inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm min^{-1}. CAT and POD activity were determined based on the method of Chance and Maehly (1955) as described in detail for creeping bentgrass in Xu and Huang (2004). Enzyme activities were based on the absorbance change of the reaction solution per minute at a given wavelength for each enzyme: CAT at 240 nm and POD at 470 nm. MDA content was measured at 532 nm and 600 nm after reaction of the extraction solution with trichloroacetic acid and thiobarbituric acid using the method of Dhindsa *et al.* (1981). The formula used for calculation of MDA content was A_{600} subtracted from A_{532} multiplied by the extinction coefficient of 155 mm^{-1} cm^{-1} for MDA (Heath and Packer, 1968). All reaction solutions, non-reacted control solutions, and standards were analysed at a given wavelength with a spectrophotometer (Spectronic Instruments, Inc., New York, NY, USA). Protein content for the activity assays was determined using the method of Bradford (1976). A 10 µl aliquot of each protein extract was mixed with 0.5 ml of dye reagent (diluted five times) (Bio-Rad Laboratories, Hercules, CA, USA). The absorbance values of each extract were measured in a spectrophotometer at 595 nm at regular intervals for 30 min. The obtained curves were compared with a standard curve developed by treating a known amount of BSA in the same fashion.

Protein extraction and quantification

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

Two-dimensional PAGE and image analysis

Protein extract samples from well-watered plants and from the 47% RWC drought stress level were run in the first dimension isoelectric focusing (IEF) by using an IPGPhor apparatus (GE Healthcare, Waukesha, WI, USA) as described in detail in Xu *et al.* (2008). Briefly, each sample contained 300 µg of protein and was subjected to IEF in immobilized pH gradient strips (pH 3.0–10.0, linear gradient, 13 cm). Following IEF, the strips were equilibrated twice for 15 min at room temperature in 50 mM TRIS-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, and 1% (w/v) DTT and then incubated with the same buffer containing 4.0% (w/v) iodoacetamide instead of DTT for 20 min. Gel electrophoresis for the second dimension was run in an SE 600 Ruby electrophoresis apparatus (GE Healthcare, Waukesha, WI, USA) in a 12.5% SDS–polyacrylamide gel. The running conditions were 5 mA per strip for 30 min followed by 20 mA per strip for 5 h. The gels were stained with Coomassie brilliant blue G-250 and scanned using a Personal Densitometer SI (63-0016-46, GE Healthcare). Gel images were scanned for relative protein content using Progenesis SameSpots software (Nonlinear Dynamics, Durham, NC, USA) with automatic default spot analysis settings including normalization with the total percentage volume of all spots on the gel to correct for potential variation due to staining. Manual correction and editing of spots where appropriate was also performed and were included in the analysis.

Protein identification and categorization

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

Experimental design and statistical analysis

The experimental design was a split-plot design with irrigation treatment as the main plots and plant materials as the subplots, with four replicates for each irrigation treatment, destructive sampling day at a given SWC, and grass type (totalling 40 plants of each plant type). The effects of watering treatment, plant materials, and corresponding interactions were determined by analysis of variance according to the general linear model procedure of SAS (version 9.0; SAS Institute, Cary, NC, USA). Differences between watering treatments and plant means were separated by Fisher's protected least significance difference (LSD) test at the 0.05 probability level.

Results

Soil water status indicating the level of soil water stress

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

Leaf physiological responses to water stress

Grass quality, EL, RWC, and F_v/F_m were not significantly different between the NT and *SAG12-ipt* plants under well-watered conditions or at the initiation of water stress treatment when SWC was maintained at 25% (Fig. 2). Leaf colour and turgidity estimated as grass quality of both NT and *SAG12-ipt* decreased in response to water stress, but at a slower rate of decline, and were maintained at a significantly higher level for *SAG12-ipt* plants than the NT plants

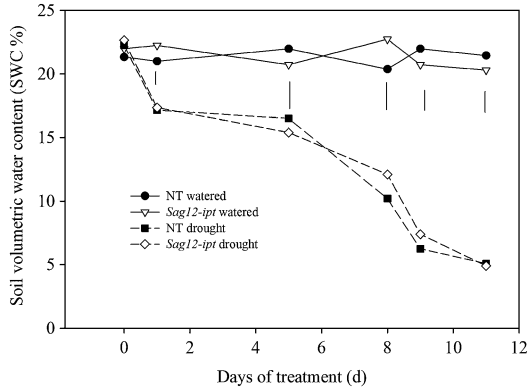

Fig. 1. Soil volumetric water content (SWC, %) measured using buried time domain reflectometry probes (20 cm) during the 14 d duration of water treatment of well-watered and drought-stressed *SAG12-ipt* and NT plants. Vertical bars indicate LSD values where significant differences were detected ($P \leq 0.05$) for comparison between plant lines on a given treatment day.

(Fig. 2A). At the end of the water stress treatment when the SWC declined to 5%, quality ratings were maintained at 4 for *SAG12-ipt* and only 1 for NT plants. Leaf EL, RWC, and F_v/F_m also declined in response to water stress in both the NT and *SAG12-ipt* plants (Fig. 2C, D). Leaf F_v/F_m declined when SWC dropped to 5% and was significantly higher in *SAG12-ipt* (0.7) than in NT plants (0.5). The decline of RWC and the corresponding increase in EL occurred at a higher SWC for NT relative to *SAG12-ipt* plants. Leaf RWC dropped to ~7% when SWC reached 10% for NT and 5% for *SAG12-ipt*.

Leaf antioxidant enzyme activity and lipid peroxidation

Under well-watered conditions at 25% SWC and throughout water stress treatments (15–5% SWC), leaf SOD, CAT, and POD had significantly higher activity in *SAG12-ipt* plants compared with NT plants (Fig. 3). SOD and POD activities were relatively unresponsive to decreasing SWC from 25% to 5% in both NT and *SAG12-ipt* leaves. CAT activity was relatively unchanged during water stress in NT leaves, but increased ~2-fold in *SAG12-ipt* leaves exposed to 5% SWC compared with that at 25% SWC. Lipid peroxidation estimated by MDA content increased during water stress, particularly for the NT plants. Leaf MDA content was 51% greater in NT compared with *SAG12-ipt* plants at 47% RWC and was greater at all levels of water stress from 15% to 5% SWC (Fig. 3D).

Root physiological responses to drought stress

Root:shoot ratios increased with decreasing SWC in both NT and *SAG12-ipt* plants, but the ratios were significantly greater in the *SAG12-ipt* plants at 10% and 5% SWC (Fig. 4A). Root MDA content increased with declining SWC from 25% to 5% in NT plants, but the increases in root MDA content did not occur until SWC decreased to 5% in *SAG12-ipt* plants (Fig. 4B). At an SWC between 25%

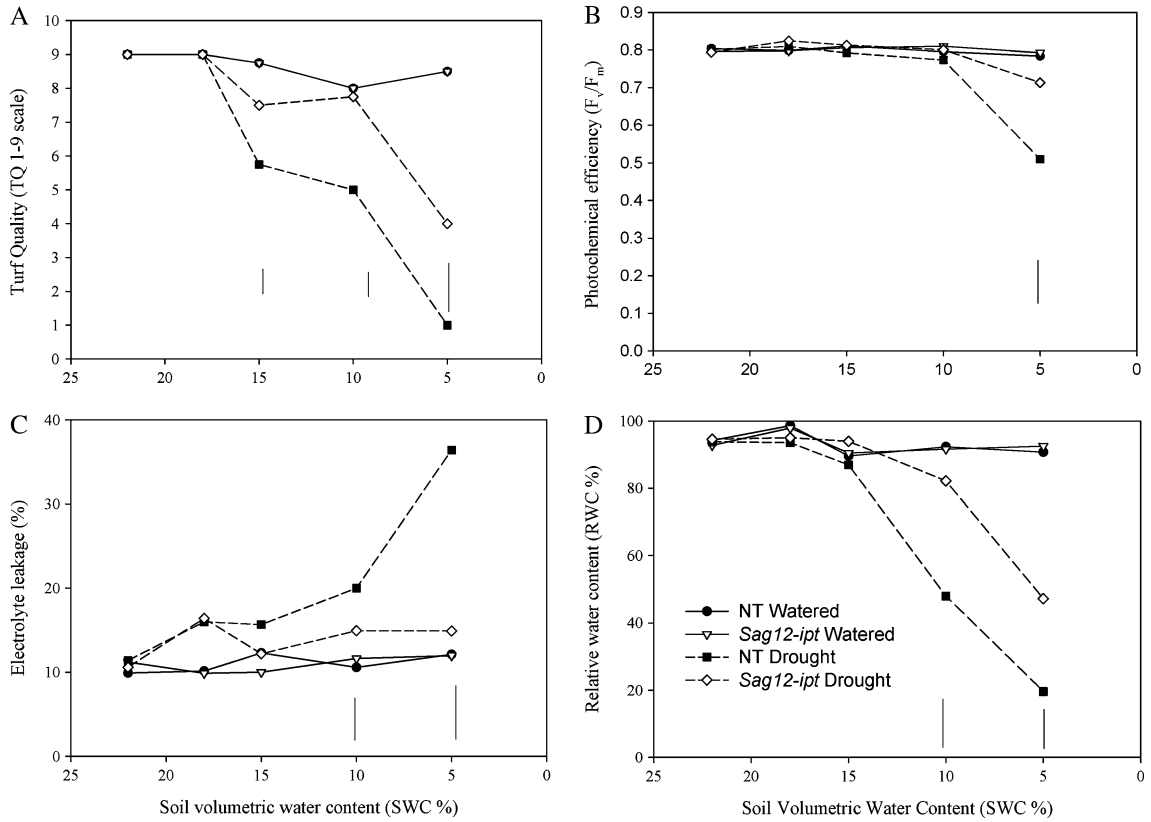

Fig. 2. Leaf physiological responses of *SAG12-ipt* and NT leaves to drought stress conditions evaluated by measurement of (A) turf quality (TQ; 1–9 scale, with 1=completely desiccated and 9=healthy, turgid), (B) photochemical efficiency (F_v/F_m), (C) relative water content (RWC, %), and (D) electrolyte leakage (EL, %). Vertical bars indicate LSD values where significant differences were detected ($P \leqslant 0.05$) for comparison between plant lines at a given soil water content (SWC %).

and 10%, the NT roots had significantly higher MDA content than those of the *SAG12-ipt* plants. Root SOD activity decreased during the decline in SWC from 25% to 5% in both NT and *SAG12-ipt* plants, but the *SAG12-ipt* plants had significantly greater SOD activity in roots than the NT roots at 25, 10, and 5% SWC (Fig. 4C). Root POD activity was relatively unchanged by decreasing SWC and was not significantly different between *SAG12-ipt* and NT roots at an SWC between 25% and 10%. At 5% SWC, POD activity was significantly higher in NT roots. No difference in CAT activity was detectable in roots of both NT and *SAG12-ipt* plants (data not shown).

Proteins exhibited differential responses to SAG12-ipt *expression and water stress*

A total of 431 protein spots were detected in each leaf sample and 315 spots were detected in each root sample. Representative gel images depicting protein spot numbers are shown in Fig. 5. A total of 64 protein spots from leaves and 83 spots from roots remain unidentified due to technical reasons such as insufficient quantity in the gel for identification. These spots are labelled with 'u' followed by a number in the gel images and will not be discussed further. The specific proteins in leaves (Table 1) or roots (Table 2) either responsive to water stress (decreased or increased abundance compared with the well-watered control columns 2 and 3) or altered by the transgene expression (different abundance levels from the water-stressed NT plants in column 4) were identified and were placed into the following categories: metabolism, energy, cell growth/division, protein synthesis, protein destination/storage, cell structure, signal transduction, disease/stress defence, secondary metabolism, and unclear (unknown function or unsuccessful identification).

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

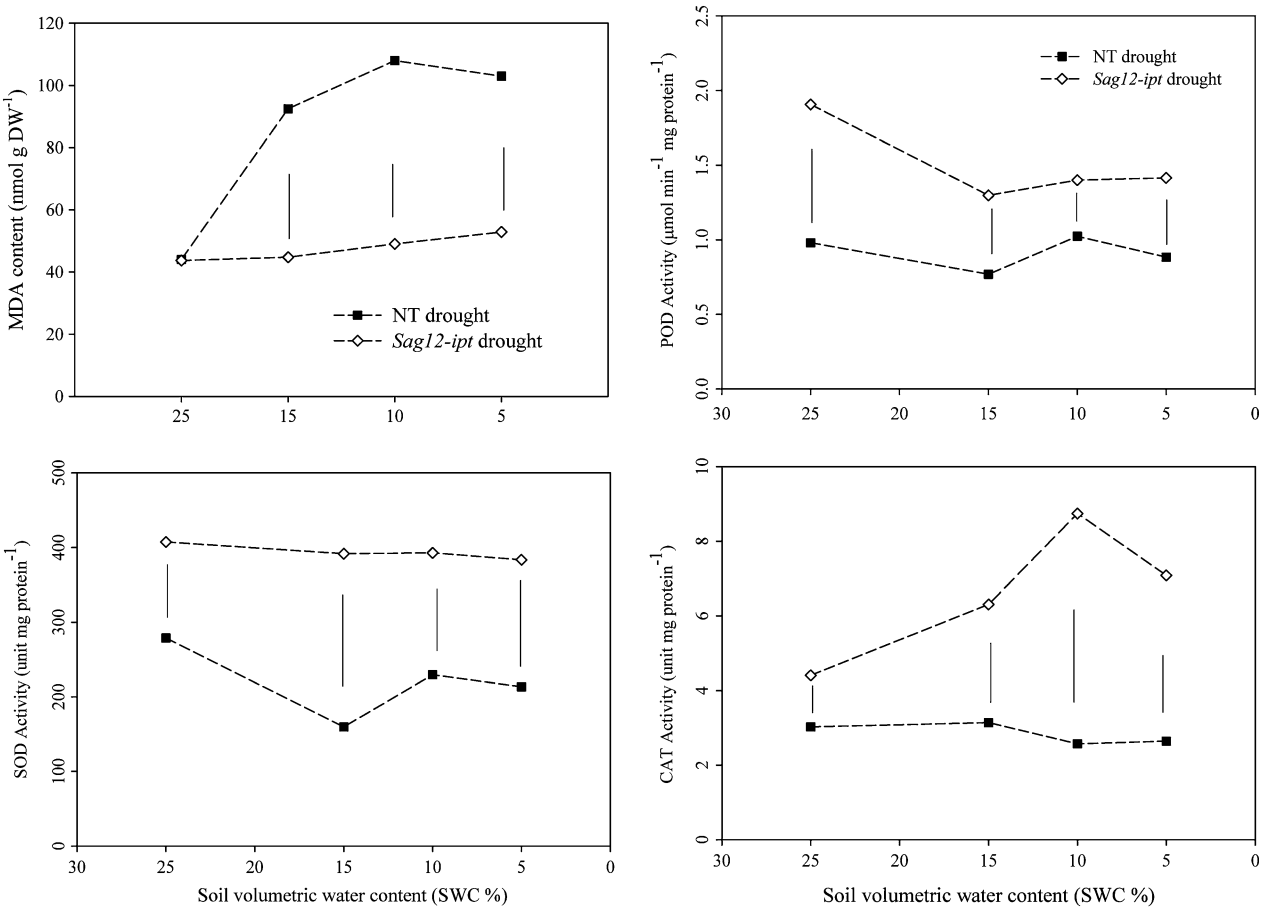

Fig. 3. Leaf antioxidant activity responses of (A) superoxide dismutase (SOD), (B) peroxidase (POD), and (C) catalase (CAT) in *ipt* transgenic creeping bentgrass (*SAG12-ipt*) compared with null transformant (NT) lines under drought stress. Vertical bars indicate LSD values where significant differences were detected ($P \leqslant 0.05$) for comparison between plant lines at a given soil water content (SWC %).

Specific proteins responsive to SAG12-ipt *expression under non-stress conditions*

Protein changes due to the presence of the transgene were determined by comparing proteins present in the two-dimensional gels derived from the well-watered control of NT with those of *SAG12-ipt* plants. In leaves, the abundance of 12 proteins was significantly higher in *SAG12-ipt* plants compared with NT, and 11 spots were identified (Table 1, column 1). These included seven proteins in the energy category, chloroplastic and cytosolic forms of glyceraldehyde phosphate dehydrogenase (GAPDH; leaf 11, 49, L36), two isoforms of the ribulose 1,5-bisphosphate carboxylase (RuBisCO) small subunit (leaf 29, 30), photosystem I subunit (PSI subunit K; PSAK) (leaf 88), and a putative phosphogluconate dehydrogenase (6PGDH; leaf L31); one protein in the protein destination/storage category [OSJNBa0039C07.4 (L34)]; two proteins involved in stress defence [CAT isoforms (leaf 111, L23)], and one with unknown function (leaf L32). The abundance of five proteins was lower in *SAG12-ipt* leaves relative to NT leaves under well-watered conditions (Table 1, column 1), of which four were identified. They were all in the energy category, including a RuBisCO small subunit (leaf 28), a chloroplastic aldolase (leaf 63), and the ATPase β-subunit (leaf 76, 77).

In roots, the abundance of 10 proteins was higher in *SAG12-ipt* plants relative to the NT line under well-watered conditions (Table 2, column 1). Of these protein spots, eight were identified, including one in metabolism (a nucleotide-sugar dehydratase), four in energy [two forms of GAPDH (root 53/R13, R14), and two forms of isocitrate dehydrogenase (IDH; root 78, 79)], one in protein synthesis (a putative asparagine-tRNA ligase, root 57), one in secondary metabolism (UDP-glucose 6-dehydrogenase, root 68), and one unknown (root R16). The abundance of five proteins was significantly lower in *SAG12-ipt* relative to NT, and three of these were identified. These included one in protein destination/storage [a protein disulphide isomerase 3 (PDI3) precursor, root 90], one in energy (a ferredoxin-nitrite reductase precursor, root R44), and one unknown (root R16).

Specific proteins responsive to SAG12-ipt *expression under water stress*

When compared as a percentage of the control, the abundance of 12 proteins increased and of 39 decreased in leaves of water-stressed NT plants (Fig. 5 and Table 1, column 2). In *SAG12-ipt* leaves, 16 protein spots exhibited increased abundance and 23 had decreased abundance

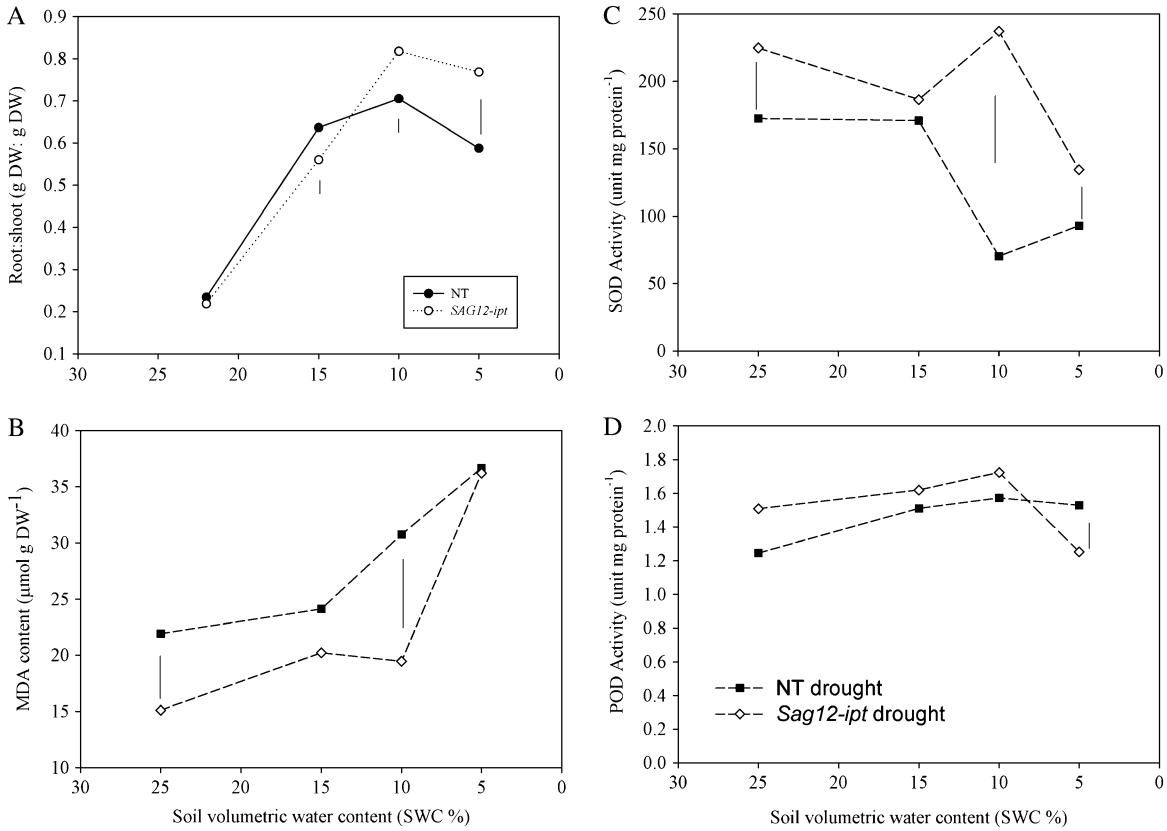

Fig. 4. Root characteristics and enzyme activity assays of *ipt* transgenic creeping bentgrass (*SAG12-ipt*) compared with null transformant (NT) lines under drought stress as measured by (A) root:shoot ratio, (B) root viability, (C) superoxide dismutase (SOD), and (D) peroxidase (POD). Vertical bars indicate LSD values where significant differences were detected ($P \leqslant 0.05$) for comparison between plant lines at a given soil water content (SWC %).

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

In the roots, the abundance of 25 protein spots increased under water stress and that of 28 proteins decreased relative to the control condition in NT plants (Fig. 5 and Table 2, column 2). In *SAG12-ipt* roots, the abundance of 22 protein spots increased and of 13 decreased under water stress (Fig. 5, and Table 2, column 3). Comparing root protein changes between *SAG12-ipt* and NT plants (comparing column 2 with 3, Table 2), 29 proteins had the same trend in accumulation in response to water stress (significantly greater or lower accumulation) whereas 54 proteins had differential accumulation in response to water stress (either unchanged, increased, or decreased in one line, but not the other, or with the opposite trend) in NT and *SAG12-ipt* roots. When comparing both plant lines under water stress (Table 2, column 4), 13 proteins had greater and 17 proteins had lower abundance in *SAG12-ipt* relative to NT plants.

Discussion

Physiological characterization of improved drought tolerance in SAG12-ipt *transgenic plants*

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

The water depletion rate, as indicated by changes in SWC content (Fig. 1) during water stress, was similar

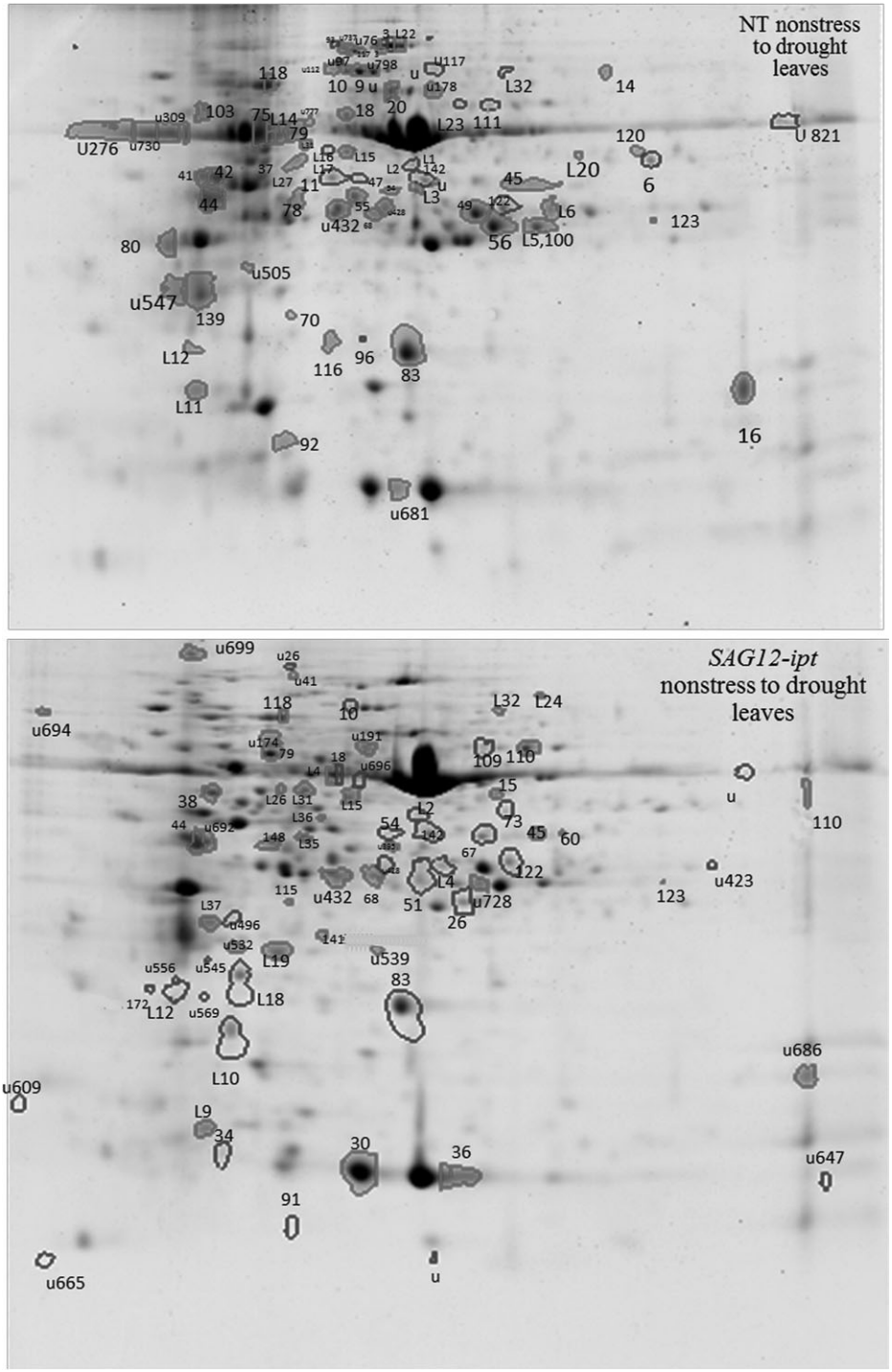

Fig. 5. Representative gel image following two-dimensional PAGE analysis of leaf protein extracts of null transformant (NT) and *ipt* transgenic creeping bentgrass (*SAG12-ipt*) exposed to water stress. Protein spots circled had differential accumulation due to water stress relative to the respective non-stressed control plant line (blue, greater accumulation; red, lower accumulation) ($P \leqslant 0.05$).

between the NT and *SAG12-ipt* plants, but at the same level of SWC and RWC (i.e. 47% RWC), transgenic plants maintained lower EL and MDA content. These data suggested that the improved shoot and root growth in the *SAG12-ipt* plants under water stress was not related to avoidance mechanisms such as a differential water depletion rate, but rather could be due to enhanced tolerance mechanisms, which is supported by the analysis of antioxidant enzyme activities. Leaf SOD, CAT, and POD activity and root SOD activity were significantly higher in *SAG12-ipt* plants compared with those in the NT plants. The tolerance mechanism most directly affected by the *ipt* gene may be increased antioxidant activity, rather than the direct regulation of water loss and cellular dehydration.

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

SID	Protein name	Transgene effect under non-stress conditions (% change in watered *Sag12-ipt* from NT)	Effects of drought (% change from control)		Differences between lines under drought (% change in drought treated *Sag12-ipt* from NT)
			NT	*SAG12-ipt*	
Category 01 Metabolism					
3	Glycine decarboxylase P subunit/Victorin binding protein [*A. thaliana*]	ns	59.5	ns	ns
4	Alanine aminotransferase [*A. thaliana*]	ns	ns	ns	10.6
6	Aminomethyltransferase [*O.sativa* (japonica cultivar-group)]	ns	47.3	ns	ns
9	Methionine synthase [MS, *Hordeum vulgare* subsp. vulgare]	ns	-30.9	ns	ns
10	Methionine synthase [MS, *H. vulgare* subsp. vulgare]	ns	-27.1	89.9*	ns
11	Aspartate aminotransferase [*O. sativa*]	ns	ns	ns	61.9
12	Cell wall beta-glucosidase (β-D-glucan exohydrolase) [*Secale cereale*]	ns	ns	ns	-31.4
13	Cell wall beta-glucosidase (β-D-glucan exohydrolase) [*Triticum aestivum*]	ns	ns	ns	-75.0
14	Cell wall beta-glucosidase isoenzyme (Beta-D-glucan exohydrolase) [*T. aestivum*]	ns	-20.2	ns	-38.7
15	UDP-sulfoquinovose synthase [*O. sativa* ('japonica' group)]	ns	ns	-78.1	34.6
16	Adenosine diphosphate glucose pyrophosphatase [*H. vulgare* subsp. vulgare]	ns	-41.4	ns	71.7
L1	Possible: 3-hydroxy-3-methylglutaryl coenzyme A reductase [*Malus x domestica*] (40 kD, pI 8.0, gil71159371)	ns	ns	ns	29.7
L24	Possible: Aspartate aminotransferase [*Pinus pinaster*] (53 kD, pI 7.5, gil59932915)	ns	ns	-45.4	ns
Y110	Glycolate oxidase	ns	ns	-43.5	ns
Category 02 Energy					
18	RuBisCO large subunit [*Psathyrostachys fragilis* subsp. Fragilis]	ns	-20.2	45.2*	ns
20	RuBisCO large subunit [*Bulbine succulenta*]	ns	-30.9	ns	ns
26	RuBisCO large subunit [*Aira praecox*]	ns	ns	34.2	56.1
28	RuBisCO small subunit [*Avena maroccana*]	-24.6	ns	ns	ns
29	RuBisCO small subunit [*T. aestivum*]	35.0	ns	ns	ns
30	RuBisCO small subunit [*T. aestivum*]	50.6	ns	-16.1	ns
32	RuBisCO small subunit [*A. maroccana*]	ns	ns	ns	-41.3
34	RuBisCO small subunit [*Bromus catharticus*]	ns	ns	19.2	ns
36	RuBisCO small subunit [*T. aestivum*]	ns	ns	-30.5	ns
38	RuBisCO activase [*Nicotiana tabacum*]	ns	ns	-20.1	72.7
41	RuBisCO activase 1 [*Gossypium hirsutum*]	ns	-67.8	ns	ns
42	RuBisCO activase 1 [*G. hirsutum*]	ns	-32.0	ns	ns
44	Phosphoribulokinase (Phosphopentokinase) [*O. sativa* ('japonica' group)]	ns	-9.9	-33.1*	ns
11	GAPDH B, chloroplast precursor [*O. sativa* ('japonica group)]	117.0	ns	ns	ns
45	GAPDH, cytosolic [*O. sativa* ('japonica group)]	ns	-28.5	-21.2	ns
47	GAPDH B, chloroplast precursor [*O. sativa* ('japonica group)]	ns	49.8	ns	ns
49	GAPDH, cytosolic [*O. sativa* ('japonica group)]	31.8	-30.9	ns	26.0
51	GAPDH A, chloroplast [*O. sativa* ('japonica group)]	ns	ns	26.3	ns
54	GAPDH, cytosolic [*O. sativa* ('japonica group)]	ns	-37.4	48.3*	ns
55	GAPDH A, chloroplast [*O. sativa* ('japonica group)]	ns	-33.9	ns	ns
56	GAPDH A, chloroplast [*O. sativa* ('japonica group)]	ns	-69.1	ns	ns
57	GAPDH A, chloroplast [*O. sativa* ('japonica group)]	ns	ns	ns	37.0
58	GAPDH B, chloroplast precursor [*O. sativa* ('japonica group)]	ns	ns	ns	-40.7
60	Cytoplasmic fructose-biphosphate (FBP) aldolase [*O.sativa*]	ns	ns	-32.1	-14.2
61	Cytoplasmic aldolase [*O. sativa*]	ns	ns	ns	-27.8
63	Chloroplastic aldolase [*O. sativa*]	-23.5	ns	ns	ns

Table 1. *Continued*

SID	Protein name	Transgene effect under non-stress conditions (% change in watered *Sag12-ipt* from NT)	Effects of drought (% change from control)		Differences between lines under drought (% change in drought treated *Sag12-ipt* from NT)
			NT	*SAG12-ipt*	
67	Cytoplasmic aldolase [*O. sativa*]	ns	ns	7.9	18.4
68	Ferredoxin-NADP(H) oxidoreductase [*T. aestivum*]	ns	-17.3	-22.6	ns
70	Triosephosphate isomerase, chloroplast precursor [*O. sativa* ('japonica group)]	ns	-69.9	ns	-63.3
72	Class III Alcohol dehydrogenase [*O. sativa*]	ns	ns	ns	22.3
73	Hydroxypyruvate reductase [*O. sativa* ('japonica group)]	ns	ns	22.7	ns
75	ATPase, β subunit [*H. vulgare*]	ns	-30.6	ns	ns
76	ATPase, β subunit [*H. vulgare*]	-35.7	ns	ns	ns
77	ATP synthase subunit β [*O. sativa* ('japonica group)]	-55.6	ns	ns	ns
78	ATP synthase □ chain [*O. sativa* ('japonica' group)]	ns	-21.9	ns	38.6
79	Enolase (2-phosphoglycerate dehydratase) [*O. sativa* ('japonica' group)]	ns	-42.8*	-24.0	ns
80	Oxygen-evolving complex protein 1 (OEE1) [*A. thaliana*]	ns	-27.1	ns	ns
83	OEE2, chloroplast precursor [*Oryza sativa* ('japonica' group)]	ns	-17.3	13.8*	ns
88	PSAK (PS I Subunit K) [*A. thaliana*]	81.6	ns	ns	ns
89	PSI subunit N, chloroplast precursor (PSI-N) [*A. thaliana*]	ns	ns	ns	67.4
91	PS I subunit VII [*O. sativa* ('japonica' group)]	ns	ns	195.8	ns
92	Cytochrome b6-f complex iron-sulfur subunit, chloroplast precursor (Rieske iron-sulfur protein) [*A. thaliana*]	ns	-50.7	ns	ns
93	Aconitate hydratase, cytoplasmic (Aconitase) [*O. sativa* ('japonica' group)]	ns	162.8	ns	36.2
96	Carbonic anhydrase, chloroplast precursor	ns	103.0	ns	ns
L10	Light-harvesting complex I; LHC I [*H. vulgare*] (24kD, pI 8.1, gil544700)	ns	ns	48.6	ns
L14	atp1 [*T. aestivum*] (55kD, pI 5.7, gil81176509)	ns	-36.7	25.3*	47.3
L15	Isocitrate dehydrogenase [NADP], chloroplastic precursor (48kD, pI 6.2, gil2497259)	ns	-42.2	-20.1*	ns
L18	PSI type III chlorophyll a/b-binding protein (29kD, pI 8.6, gil430947)	ns	ns	80.1	ns
L19	Triosephosphate isomerase, cytosolic; (27kD, pI 5.4, gil2507469)	ns	ns	-28.4	ns
L2	Possible: putative cytochrome c oxidase subunit IIPS17 (2kD, pI9.6, gil109892850)	ns	76.7	91.8*	18.7
L20	6-phosphogluconate dehydrogenase, decarboxylating [*Chlamydomonas reinhardtii*] (61 kD, pI8.4, gil15225026)	ns	-43.1	ns	ns
L31	Putative phosphogluconate dehydrogenase [*O. sativa* 'japonica' group] (45kD, pI5.4, gil55295906)	13.2	-20.2	-20.7	ns
L36	Glyceraldehyde 3-phosphate dehydrogenase β subunit [*A. thaliana*] (43kD, pI5.6,gil336390)	71.9	ns	-39.3	ns
L4	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (33kD, pI 6.2, gil120668)	ns	ns	67.6	ns
L6	Possible: ferredoxin [*Zea mays*] (41kD, pI 8.7, gil162458489)	ns	-34.4	ns	ns
L7, 135	Photosystem I subunit VII [*Oryza sativa* 'japonica' group] (8.9kD, pI6.5)	ns	ns	147.5	19.6
L9	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [*Avena sativa*] (18kD, pI 8.8, gil4038695)	ns	ns	-33.7	ns
Y172	Chloroplast chlorophyll a/b-binding protein precursor [*Oryza sativa*]	ns	ns	125.1	ns

Table 1. *Continued*

SID	Protein name	Transgene effect under non-stress conditions (% change in watered *Sag12-ipt* from NT)	Effects of drought (% change from control)		Differences between lines under drought (% change in drought treated *Sag12-ipt* from NT)
			NT	*SAG12-ipt*	
Category 03 Cell growth/division					
L16	Possible: DEAD-box ATP-dependent RNA helicase 2 -Arabidopsis thaliana (Mouse-ear cress) (46kD, pI 6.0, gil109893655)	ns	26.9	ns	ns
Category 05 Protein synthesis					
120	Chloroplast translational elongation factor Tu [*O. sativa* 'japonica' group] 50kD, pI6.1)	ns	-50.9	ns	ns
L5	Putative RNA binding protein [*A. thaliana*] (43kD, pI 8.2, gil3850621)	ns	-19.9	ns	ns
L17	Possible:mitochondrial elongation factor Tu [*A. thaliana*] (52 kD, pI 5.5,gil1 149571)	ns	57.6	ns	ns
L30	Possible: Elongation factor G, chloroplast precursor (ISS),[*Ostreococcus tauri*] (86kD, pI5.3, gil1 16059008)	ns	ns	ns	-40.0
Category 06: Protein destination/storage					
101	Heat shock protein 70 [*Cucumis sativus*]	ns	ns	ns	-26.9
103	RuBisCO large subunit-binding protein subunit beta, (60 kDa chaperonin subunit beta)	ns	-33.9	ns	ns
L3	Putative protein disulphide isomerase (PDI) [*Brassica napus* var. napus] (26kD, pI 6.5, gil45593261)	ns	-42.5	ns	ns
L34	OSJNBa0039C07.4 (HSP93 III) [*O. sativa* ('japonica' group)] 98kD, pI5.8, gil38347158)	14.3	ns	ns	-39.5
Category 09 Cell structure					
L35	Type IIIa membrane protein cp-wap13 [*Vigna unguiculata*] (39kD, pI6.2, gil2218152)	ns	ns	-33.5	ns
Category 11 Disease/defense					
109	Catalase-1 [*O. sativa* ('japonica' group)]	ns	ns	18.1	19.0
110	Catalase-1 [*O. sativa* ('japonica' group)]	ns	ns	-25.9	ns
111	Catalase-1[O. *sativa* ('japonica' group)]	58.7	45.7	ns	ns
114	Ascorbate peroxidase APX4 [*A. thaliana*]	ns	ns	ns	20.8
115	Ascorbate peroxidase APX7, chloroplastic [*O. sativa* ('japonica' group)]	ns	ns	ns	-23.6
116	Glutathione-S-transferase (GST) [*H. vulgare*]	ns	-40.6	ns	17.1
L11	2-Cys peroxiredoxin BAS1, chloroplast precursor [*T. aestivum*] (23kD, pI 5.7, gil2829687)	ns	-31.3	ns	ns
L23	Possible: catalase (56kD, pI 6.7, gil1705626)	26.6	87.2	ns	ns
L37	Possible: ascorbate peroxidase [*A. thaliana*] (28kD, pI5.9, gil555576)	ns	ns	-20.6	ns
Category 20 Secondary metabolism					
L22	Glycine decarboxylase P subunit [*Tritordeum* sp.] (111kD,pI6.5, gil2565305)	ns	-67.8	ns	ns
L27	S-adenosylmethionine synthetase (gil3914019)	ns	-37.7	ns	ns
Unclear					
111	Unknown	ns	-27.1	ns	ns
118	Unknown	ns	-37.4	-15.5	ns
119	Unknown	ns	ns	ns	-59.9
122	Unknown	ns	35.3	30.9	-19.5
123	Unknown	ns	-37.7	-50.4	ns
127	Unknown	ns	ns	ns	15.0
131	Unknown	ns	ns	ns	69.3
137	Unknown	ns	ns	ns	69.3

Table 1. *Continued*

SID	Protein name	Transgene effect under non-stress conditions (% change in watered *Sag12-ipt* from NT)	Effects of drought (% change from control)		Differences between lines under drought (% change in drought treated *Sag12-ipt* from NT)
			NT	*SAG12-ipt*	
139	Unknown	ns	-30.9	ns	ns
141	Unknown	ns	ns	22.2	ns
142	Unknown	ns	52.8*	27.7	19.6
148	Unknown	ns	ns	-26.2	44.1
L12	Unknown	ns	-68.1	261.2*	60.1
L26	Unknown	ns	ns	-33.1	ns
L32	Unknown	77.1	47.0*	-36.3	ns

Proteomic changes associated with SAG12-ipt *expression and water stress*

The expression of *ipt* in creeping bentgrass caused changes in protein abundance under both well-watered and water stress conditions. The differential protein expression in *SAG12-ipt* compared with NT in well-watered plants was presumably due to *ipt* expression associated with natural leaf senescence. PCR-based transcript expression of *SAG12-ipt* was detected under non-stressed conditions previously in creeping bentgrass (Merewitz *et al.*, 2011), and similar findings have been reported in petunia (*Petunia×hybrida*) (Clarke, *et al.*, 2004), maize (*Zea mays*) (Robson *et al.*, 2004), and tobacco (Rivero *et al.*, 2007). The *ipt* expression under non-stress conditions was associated with higher levels of isopentenyl adenine (iPa) in immature leaves, mature leaves, and roots, and higher zeatin riboside (ZR) in mature leaves of creeping bentgrass (Merewitz *et al.*, 2011). The differential protein accumulation in non-stressed NT and *SAG12-ipt* plants, as described in the Results above, is due to the elevated CKs and expression of *ipt* associated with natural leaf or root senescence.

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

Protein changes within the metabolism category

Differential responses in the metabolic enzymes in both leaves and roots between the NT and transgenic plants were primarily related to enzymes involved in amino acid and cell wall degradation or biosynthesis. Changes in amino acid content have downstream effects on protein synthesis and other stress responses such as osmotic adjustment (OA). Cell wall-modifying enzymes may affect cell wall elasticity, thereby regulating cell turgor (Bohnert and Jensen, 1996). The abundance of two proteins related to methionine metabolism, methionine synthase (MS; leaf 9, 10; root 73) and *S*-adenosylmethionine synthetase (SAMS; leaf L27; root 40, 41, R18), was increased and unchanged, respectively, in *SAG12-ipt* in response to water stress, whereas their accumulation decreased in leaves and roots of the NT plants. The activation of MS is an early response to drought symptoms since increased flux through the pathway provides a source of carbon under stress. During severe stress, MS activity declines. SAMS is downstream of MS and can be a source of methyl groups for key secondary metabolites such as osmoprotectants involved in OA (Bohnert *et al.*, 1996). Thus, the increase or maintenance of MS and SAMS content in *SAG12-ipt* plants may reflect more active methionine and osmoregulant metabolism than in the NT plants under water stress. Similarly, induction of MS transcripts and an increase in MS protein content under salt stress has been associated with salt stress tolerance in barley (*Hordeum vulgare*) (Narita *et al.*, 2004).

Other proteins in the metabolism category that were differentially accumulated within the plant lines, such as aspartate and alanine aminotransferases, also may be involved in OA as well as in the activation of antioxidant enzymes to reduce the amount of reactive oxygen species (ROS) generated by drought stress (Kocsy *et al.*, 2005). Increased levels of aspartate aminotransferase in *SAG12-ipt* plants (leaf 11) may allow for increased OA under water stress conditions, but, since the other isoform of this enzyme (leaf L24) was reduced by water stress in *SAG12-ipt*, the trend in accumulation of this enzyme is unclear. The accumulation of these enzymes is highly dependent on the level of free precursors for aspartate synthesis (Good and Zaplachinski, 1994). In roots, glutamine synthetases (GSs)

accumulated more in *SAG12-ipt* than in NT in response to water stress (root 4, 71, 72). Root cytosolic GS is involved in the assimilation of ammonia, N transport/remobilization, and control of root biomass, and GS content/activity typically decreases in response to drought stress (Bauer *et al.*, 1997; Limami *et al.*, 1999). Increased expression of GS genes contributes to drought and salt tolerance (Kalamaki *et al.*, 2009). This is consistent with the increased levels of N-metabolizing enzymes such as IDH reported here and discussed in the energy section. Increased flux through N metabolic pathways suggests an enhancement of N uptake by the roots under stress, which is important for plant stress tolerance.

Some leaf proteins involved in metabolism were at lower levels in *SAG12-ipt* plants compared with NT plants, including isoforms of cell wall β-glucosidase (β-D-glucan exohydrolase) (leaf 12, 13, 14), glycine decarboxylase P subunit/Victorin-binding protein (leaf 3), aminomethyltransferase (leaf 6), and glycolate oxidase (leaf Y110). Cell wall β-glucosidases may have a role in cell wall reinforcement (Ricardi *et al.*, 1998; Dietz *et al.*, 2000; Caruso *et al.*, 2009); however, the role of β-glucosidase in drought stress is not well understood. β-Glucosidases are also implicated in the release of active CKs and abscisic acid (ABA) from inactive forms during stress. Perhaps lower CK content in NT plants and more severe stress led to increased levels of this enzyme in order to release active CK to a greater extent than in *SAG12-ipt* plants. Glycine decarboxylase and aminomethyltransferase are involved in the breakdown of glycine, which when present in the form of glycine betaine is involved in OA during drought stress (Chen and Murata, 2008). Since maintenance of turgor and OA are tightly linked, lower levels of these proteins under water stress in *SAG12-ipt* plants compared with NT plants at the same level of RWC could indicate a reduction in cellular damage in *SAG12-ipt* plants, thereby requiring less cell wall modification and OA to maintain turgidity. Glycolate oxidase is typically induced by drought stress since it is a key factor in photorespiration and may increase endogenous H_2O_2 (Ingram and Bartels, 1996). Greater levels of this protein in NT plants may also indicate that NT plants were experiencing greater stress damage than *SAG12-ipt* plants.

Protein changes within the energy production category

In leaves, photosynthetic proteins such as RuBisCO large subunits accumulated to a greater extent in *SAG12-ipt* leaves (leaf 18, 20, 26), whereas these proteins either significantly declined or were unchanged in NT leaves in response to water stress relative to their respective control plants. The greater levels of RuBisCO subunits in leaves in *SAG12-ipt* bentgrass under well-watered and water stress conditions are consistent with results found in *ipt* transgenic tobacco under non-stressed conditions (Rivero *et al.*, 2009) and *SAG12-ipt* creeping bentgrass under heat stress (Y Xu *et al.*, 2010), which showed higher levels of RuBisCO transcripts than the non-transgenic plants. The increase in the abundance of RuBisCo large subunits and the decrease in small subunits have also been found in non-transgenic wheat in response to drought (Caruso *et al.*, 2009). Other proteins involved in photosynthesis that were generally greater in *SAG12-ipt* under water stress than in NT relative to their respective controls were chloroplast precursors (leaf 83, 89, 92, Y172) or those involved in the electron transport chain such as PSI proteins (leaf 88, 91, L18, L7/135), oxygen-evolving complexes (OEEs; spots 80, 83), cytochrome complexes (leaf 92, L2), and a ferredoxin (leaf L6). A reduction in the rate of chlorophyll degradation in *SAG12-ipt* leaves under water and heat stress (Merewitz *et al.*, 2010, 2011; Y Xu *et al.*, 2010) and the maintenance of F_v/F_m and chloroplast proteins demonstrated in this study are likely to be determinants of sustained CK action under stress, since adequate chloroplast development is necessary for CKs to elicit a growth response in leaves (Kulaeva *et al.*, 2002).

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

Proteins involved in respiration pathways such as glycolysis were responsive to both the *SAG12-ipt* transgene and water stress in both leaves and roots. The majority of GAPDH isoforms detected were elevated in *SAG12-ipt* plants in both leaves (leaf 11, 45, 47, 49, 51, 54, 55, 56, 57, L4) and roots (root R3, 53/R15, R14) under water stress conditions, whereas the abundance of GAPDH either remained unchanged or decreased in response to water stress in NT plants. GAPDH catalyses a key step in glycolysis that breaks down glucose into carbon and energy. The higher levels of the cytosolic form of GAPDH in the *SAG12-ipt* plants relative to NT plants under well-watered conditions may reflect less glycolysis characteristic of natural leaf senescence and may predispose *SAG12-ipt* plants to enhanced tolerance. Under stress, it has been found that GAPDH transcription and protein abundance levels increased in some plant species (Yang *et al.*, 1993; Chang *et al.*, 2000; Ferreira *et al.*, 2006). GAPDH may increase in response to stress initially, since it is often an immediate response to drought stress (Ingram and Bartels, 1996), and then decline as cellular damage and proteolytic activity increase. Velasco *et al.* (1994) showed that extremely drought-tolerant resurrection plants exhibited up-regulation of the cytosolic form of GAPDH transcripts, and

Table 2. Effects of the transgene and drought stress on protein abundance in roots of *SAG12-ipt* and NT creeping bentgrass

SID	Protein name	Transgene effect under non- stress conditions (% change in watered *Sag12-ipt* from NT)	Effects of drought (% change from control)		Differences between lines under drought (% change in drought treated *SAG12-ipt* from NT)
			NT	*SAG12-ipt*	
Category 01 Metabolism					
4	Cytosolic glutamine synthetase (EC 6.3.1.2) [*Populus alba x Populus tremula*]	ns	-4.5	92.0*	ns
6	Serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1) [*Arabidopsis thaliana*]	ns	-33.2	ns	ns
7	Nucleotide-sugar dehydratase [*Arabidopsis thaliana*]	9.7	ns	ns	-31.3
48	Phosphoserine aminotransferase (EC 2.6.1.52) [*O. sativa*]	ns	ns	12.0	ns
51	Plastidic ATP sulfurylase (APS) (EC 2.7.7.4) [*O. sativa*]	ns	191.0	ns	-27.1
71	Cytosolic glutamine synthetase [*Glycine max*]	ns	ns	ns	25.5
72	Cytosolic glutamine synthetase [*Populus alba x Populus tremula*]	ns	109.6	147.6*	73.4
73	Methionine synthase [*Hordeum vulgare* subsp. vulgare]	ns	-32.8	ns	ns
R21	Cytosolic 6-phosphogluconate dehydrogenase [*Oryza sativa*] (52kD, pI 6.5, gil38426301)	ns	ns	-22.2	ns
R20	Possible: Os08g0459600 [*O. sativa* 'japonica'] (44D, pI 8.7, gil1 15476758)	ns	-28.4	74.1*	ns
R26	Possible: UCW116, putative lipase [*H. vulgare* subsp. vulgare] (39kD, pI7.4, gil1 18748148)	ns	ns	ns	27.4
R43	Possible: aspartyl-tRNA synthetase [*Zea mays*] (61kD, pI 5.9, gil226505476)	ns	11.8	ns	ns
Category 02 Energy					
8	Cytoplasmic aconitate hydratase (EC 4.2.1.3) [*A. thaliana*]	ns	-28.5	-37.0	ns
12	Pyruvate kinase (EC 2.7.1.40) [*Glycine max*]	ns	23.6	ns	ns
20	Ferredoxin-NADP reductase precursor (EC 1.18.1.2) [*Zea mays*]	ns	ns	-37.2	ns
21	Ferredoxin-NADP reductase precursor (EC 1.18.1.2) [*Zea mays*]	ns	81.7*	35.9	-35.1
52	Sucrose synthase (EC 2.4.1.13) Ss1 [*H. vulgare*]	ns	-43.8	ns	ns
53	Cytosolic GAPDH (phosphorylation) (EC 1.2.1.12) [*H. vulgare*]	14.2	35.5	ns	ns
54	GAPDH (phosphorylating) (EC 1.2.1.12) [*H. vulgare*]	ns	ns	72.4	ns
55	Cytoplasmic FBP aldolase (EC 4.1.2.13) [*O. sativa*]	ns	-29.7	-37.3	ns
78	Isocitrate dehydrogenase [NADP], chloroplast precursor	55.9	-17.7	ns	17.8
79	Isocitrate dehydrogenase [*O. sativa* ('japonica' group)]	31.3	ns	ns	ns
81	Triosephosphate isomerase, cytosolic [*T.aestivum*]	ns	ns	50.7	ns
82	Enolase (2-phosphoglycerate dehydratase) [*T. aestivum*]	ns	ns	38.7	ns
83	Enolase [*Oryza sativa ('*japonica' group)]	ns	ns	63.8	ns
R1	L-malate dehydrogenase (MDH) [*A. thaliana*] (42kD, pI 9.0, gil15232820)	ns	ns	ns	-23.0
R3	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (33kD, pI 6.2, gil120668)	ns	30.8	40.6	ns
R14	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (33kD, pI 6.2, gil120668)	17.1	ns	ns	ns
R17	NADPH producing dehydrogenase of the oxidative pentose phosphate pathway [*Zea mays*] (53kD, pI 5.9, gil162463282)	ns	-17.8	ns	-38.7
R21	Cytosolic 6-phosphogluconate dehydrogenase [*O. sativa*] (52kD, pI 6.5, gil38426301)	ns	-64.4	ns	ns
R22	Ribulose-1,5-bisphosphate carboxylase, large subunit [*Didymosalpinx norae*] (52kD, pI 6.5, gil1770216)	ns	54.0	79.7*	ns
R23	O-methytransferase 4 [*T. aestivum*] (38kD, pI 5.6, gil145693798)	ns	10.8	ns	ns
R27	Ferredoxin-NADP reductase precursor [*Z. mays*] (36kD, pI8.4,) homologue	ns	106.3	94.8	ns
R39	ATP synthase beta subunit [*T. aestivum*] (59kD, pI 4.7, gil525291)	ns	ns	148.0	77.3
R44	Ferredoxin-nitrite reductase precursor [*T. aestivum*] (66kD, pI6.9, gil218963620)	-51.9	-57.8	ns	ns
Y165	Glucose-6-phosphate isomerase (GPI) cytoplasmic (62 kD, pI 6.96, Accn P49105)	ns	ns	ns	34.7

Table 2. *Continued*

SID	Protein name	Transgene effect under non- stress conditions (% change in watered *Sag12-ipt* from NT)	Effects of drought (% change from control)		Differences between lines under drought (% change in drought treated *SAG12-ipt* from NT)
			NT	*SAG12-ipt*	
Category 05 Protein synthesis					
57	Putative asparagine-tRNA ligase.(EC 6.1.1.22) [*O. sativa*]	12.9	-69.6	-50.5*	-18.0
Category 06 Protein destination/storage					
23	Mitochondrial processing peptidase α-chain (MPP) (EC 3.4.24.64)	ns	-52.7	-31.2	ns
86	Endoplasmin homolog precursor (HSP90) [*T. aestivum*]	ns	ns	52.3	51.0
88	70 kDa heat shock cognate [*Vigna radiata*]	ns	ns	121.1	ns
90	Protein disulfide isomerase (PDI) 3 precursor [*T. aestivum*]	-53.8	-16.4	ns	ns
R6	Os09g0505600 [*O. sativa* 'japonica'] (24kD, pI 6.4) (possible proteasome function)	ns	ns	-29.7	-48.9
R29	Possible: Proteasome subunit α type-7 (28kD, pI8.4)	ns	143.1	153.6	ns
R40	Possible: heat shock protein 83 [*A. thaliana*] (81kD, pI5.0)	ns	ns	35.5	39.6
Y153	Putative t-complex protein 1 theta chain [*O. sativa*]	ns	-69.0	-50.5	ns
Category 08 Intracellular traffic					
59	Ran (Small GTP-binding protein) (Ran2) [*O. sativa*]	ns	ns	ns	-18.5
Category 09 Cell structure					
26	Reversibly glycosylated polypeptide [*T. aestivum*]	ns	52.4	40.0	ns
R46	Actin [*Cleistogenes songorica*] (42kD, pI5.5)	ns	77.0	109.0*	134.0
R47	Actin-1 (42kD, pI 5.5)	ns	99.4	133.9*	ns
Y18 6	β-5 tubulin [*Triticum aestivum*]	ns	ns	103.7	35.9
Category 10 Signal transduction					
29	GTP-binding protein [*O. sativa*]	ns	49.4	ns	ns
30	GTP-binding protein beta chain homolog curled-leaved [*N. tabacum*]	ns	43.2	ns	-22.8
31	GTP-binding protein beta chain [*N. tabacum*]	ns	ns	ns	-7.8
R49	Possible: 14-3-3E [*H. vulgare* subsp. vulgare] (29kD, pI4.8)	ns	-17.5	31.2*	ns
Category 11 Disease/defense					
33	Probable peroxidase (EC 1.11.1) 1 precursor anionic [*Z.mays*]	ns	ns	ns	-32.2
34	Probable peroxidase (EC 1.11.1) 1 precursor anionic [*Z.mays*]	ns	27.9	ns	ns
64	Superoxide dismutase (EC 1.15.1.1) (Mn) 3.2 precursor [*Z.mays*]	ns	77.3	ns	ns
66	Sti (Stress inducible protein) [*Glycine max*]	ns	ns	-32.0	-20.8
R5	Ascorbate peroxidase [*H. vulgare* subsp. vulgare] (27kD, pI 5.8)	ns	79.2	46.2	-24.3
Category 20 Secondary metabolism					
38	dDTP-glucose 4-6-dehydratases-like protein [*A. thaliana*]	ns	-16.7	ns	ns
39	Adenosylhomocysteinase (EC 3.3.1.1) [*T. aestivum*]	ns	-35.3*	-54.4	ns
40	S-adenosylmethionine synthase (SAMS) (EC 2.5.1.6) [*A. thaliana*]	ns	-31.7	-27.2	ns
41	SAMS (EC 2.5.1.6) [*Dendrobium crumenatum*]	ns	-31.0	ns	ns
68	UDP-glucose 6-dehydrogenase (EC 1.1.1.22) [*Glycine max*]	38.3	-29.1	ns	ns
93	UDP-glucose dehydrogenase [*O. sativa* 'japonica' group]	ns	-13.9	ns	ns
R18	S-adenosylmethionine synthetase (43kD, pI 5.4)	ns	-30.0	ns	33.9
Category 12 Unclear					
46	Unknown	ns	51.7	ns	ns
47	Unknown	ns	-11.7	ns	ns
94	Os04g0650800 [*O. sativa ('*japonica' group)]	ns	-31.5	ns	ns
97	Unknown	ns	34.2	ns	-45.6
98	Unknown	ns	-3.1	-39.8*	ns
99	Unknown	ns	24.3	ns	ns
101	Unknown	52.9	ns	ns	ns
102	Unknown	ns	205.0	ns	ns
104	Unknown	ns	7.0*	-26.1	-48.6
R2	Putative r40c1 protein - rice [*O. sativa* 'japonica' group] (42 kD, pI 6.2)	ns	117.9	ns	ns
R16	Unknown	-61.8	-56.3	ns	50.1
R19	unknown	ns	ns	25.5	ns

Table 2. *Continued*

SID	Protein name	Transgene effect under non- stress conditions (% change in watered *Sag12-ipt* from NT)	Effects of drought (% change from control)		Differences between lines under drought (% change in drought treated *SAG12-ipt* from NT)
			NT	*SAG12-ipt*	
R28	unknown	ns	180.8	313.8*	ns
R30	unknown	ns	74.3	211.9*	ns
R31	unknown	ns	-32.4	-43.6	ns
R32	unknown	ns	115.8	ns	ns
R33	unknown	ns	ns	93.5	-41.1
R34	unknown	ns	ns	ns	-23.0
R48	unknown	ns	80.6*	69.0	14.5
R51	unknown	ns	ns	172.0	ns

a rapid stimulation of glycolysis was an important characteristic in the drought response to maintain available energy under stress. Also, recently, GAPDH was found to be a direct target of CK action (Heintz *et al.*, 2006) and is believed to be involved in the stress defence via the antioxidant defence system by prevention of hydrogen peroxide-mediated cell death (Baek *et al.*, 2008). Promotion of photorespiratory processes was also indicated in *ipt* transgenic tobacco under water stress (Rivero *et al.*, 2009). Thus, the decline in GAPDH in NT leaves and the ability of *SAG12-ipt* plants to maintain or increase GAPDH content and glycolysis could be a significant component contributing to drought tolerance by promoting both energy production and antioxidant defence.

Other proteins involved in respiration, such as leaf and root aldolases (leaf 61, 63, 67; root 52), leaf triose phosphate isomerases (TPIs; leaf 70, L19), and leaf enolase (leaf 79), were generally unchanged or down-regulated by water stress in both *SAG12-ipt* and NT plants. Similar results for these enzymes were found in creeping bentgrass leaves under salt stress (C Xu *et al*, 2010). Interestingly, in roots, IDH content was greater in the non-stressed condition in *SAG12-ipt* than in NT plants. Under drought stress, TPI (root 81) and enolase (root 82, 83) were increased in *SAG12-ipt* but not in NT plants, and root sucrose synthase (root 52) was maintained in *SAG12-ipt* but reduced in NT plants. These differences suggest an activation of glycolysis and sugar metabolism in *SAG12-ipt* plants, which may support root growth under stress (Konishi *et al.*, 2005). Previous work has shown greater root growth and viability of *SAG12-ipt* plants under drought and heat stress conditions (Merewitz *et al.*, 2010, 2011; Y Xu. *et al.*, 2010). The present study also demonstrated a higher root to shoot ratio and lower root lipid peroxidation (MDA content). The abundance of IDH was greater in *SAG12-ipt* roots than in NT plants under non-stressed conditions and was decreased due to drought in NT roots, but not significantly changed in *SAG12-ipt* roots. IDH is an enzyme of the tricarboxylic acid (TCA) cycle, may be involved in N assimilation, has been found to be associated with leaf senescence, and its production of NADP could contribute to antioxidant defences but is dependent on the specific isoform or cellular location (Corpas *et al.*, 1999). Previously, *SAG12-ipt* plants were shown to maintain higher levels of IDH in roots under heat stress than NT plants (C Xu *et al.*, 2010). Inhibition of senescence may require more N for sustained chlorophyll and protein synthesis in maturing leaves. Since drought may restrict N uptake, and greater levels of N uptake have been associated with drought tolerance (Patrick and Wyatt, 1964; Foyer *et al.*, 1998), increased IDH prior to and during drought stress may allow *SAG12-ipt* to develop more efficient N and antioxidant metabolism for increased drought adaptability. However, the different isoforms of IDH could contribute to different metabolic functions or tolerance mechanisms; thus further work to identify changes in the different isoforms of this protein specifically in response to leaf senescence and drought in *SAG12-ipt* plants may be warranted.

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

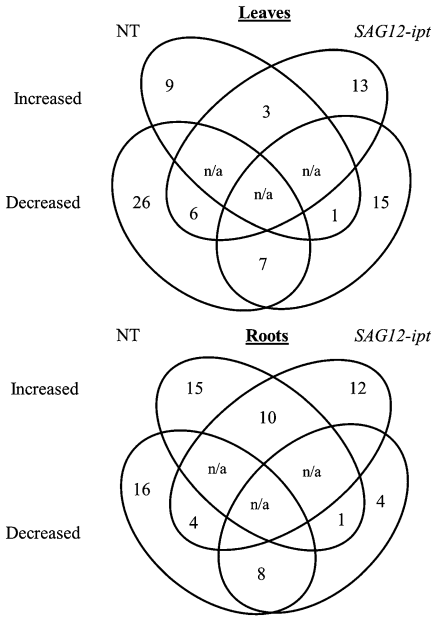

Fig. 6. Four-way Venn diagram comparing the number of proteins that exhibited a significant ($P \leq 0.05$) increase or decrease due to water stress in *ipt* transgenic creeping bentgrass (*SAG12-ipt*) compared with null transformant (NT) plant lines relative to the protein content of the respective well-watered control plants for leaves and roots. Overlapping regions of the circles indicate proteins that were regulated in either the same or the opposite manner in the respective treatment, whereas non-overlapping circles indicate proteins regulated in only that treatment.

Changes in proteins with functions related to protein synthesis

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

In roots, the abundance of a putative asparagine-tRNA ligase (root 57) accumulated more in *SAG12-ipt* relative to the change in NT in both the non-stressed and stressed conditions. Asparagine-tRNA ligase is an enzyme that catalyses the reaction determining the aminoacyl-tRNA activity state for alanine and aspartate metabolism and aminoacyl-tRNA biosynthesis. Conversion of the tRNA to the AMP form by the ligase can lead to asparagine synthesis. In regards to its possible association with the *SAG12-ipt* transgene, a delay in senescence has been linked to a delay in accumulation of asparagine and other free amino acids (Downs *et al.*, 1997). Aminoacyl-tRNA molecules are associated with other processes in addition to protein synthesis, such as the synthesis of porphyrin ring structures, phospholipid synthesis, or peptidoglycan cross-linking (Mocibob *et al.*, 2010). Since this enzyme was more abundant under non-stressed conditions in *SAG12-ipt*, it could be involved in the CK biosynthesis promoted by the *SAG12-ipt* gene. Under water stress, this enzyme may be beneficial to *SAG12-ipt* roots by stimulating biosynthesis of these molecules. For instance, phospholipid and peptidoglycans could aid in membrane and cell wall stability. In addition, the direct product of the *SAG12-ipt* gene, iPa, is directly associated with tRNAs in translation, and derivatives of iPa may improve tRNA efficiency (Persson *et al.*, 1994). In general, elevated levels of proteins involved in translation could be beneficial for maintenance of protein synthesis under drought stress and be a factor in the reduced senescence in *SAG12-ipt* plants Increased efficiency of protein synthesis under stressed conditions, when metabolic costs are high and restricted, may allow for increased metabolic functioning in *SAG12-ipt* roots.

Changes in proteins involved in the regulation of protein destination/storage

In both leaves and roots, the abundance of PDI (leaf L3 and root 90) was maintained in *SAG12-ipt* plants during water stress, but was reduced by water stress in leaves and roots of NT plants. In roots, water stress caused an increase in the endoplasmin homologue precursor of HSP90 (root 86), Hsp70 cognate (root 88), and the proteasome subunit alpha type-7 (root R29), and a decrease in a mitochondrial processing peptidase (MPP; root 23). Increases in the abundance of these proteins were most pronounced in *SAG12-ipt* roots, whereas decreases were more prominent in NT roots. Both PDI and a ferredoxin-nitrite reductase precursor were lower in *SAG12-ipt* than in NT plants under well-watered conditions. The abundance of root Hsp83 (R40) was increased by drought in response to water stress in *SAG12-ipt*, but a significant change did not occur in NT. Thus, improvements in drought tolerance of *SAG12-ipt* relative to NT could be related to increased protein chaperone and import function capabilities since Hsp90, Hsp70, and PDI are all involved in assisting protein folding (Georgopoulos and Welch, 1993). Similarly, MPP is involved in protein import to (Braun and Schmitz, 1997) and replacement of damaged proteins in the mitochondria during stress conditions (Taylor *et al.*, 2005).

Changes in proteins functioning within the cellular structure and growth category

One leaf protein in the cell structure category, a type IIIa membrane protein cp-wap13 (leaf L35), exhibited differential accumulation in response to water stress between the *SAG12-ipt* and NT plants. Cp-wap13 proteins are associated with the Golgi apparatus as well as cellulose biosynthesis in the cell wall, primarily in plasmodesmata, and

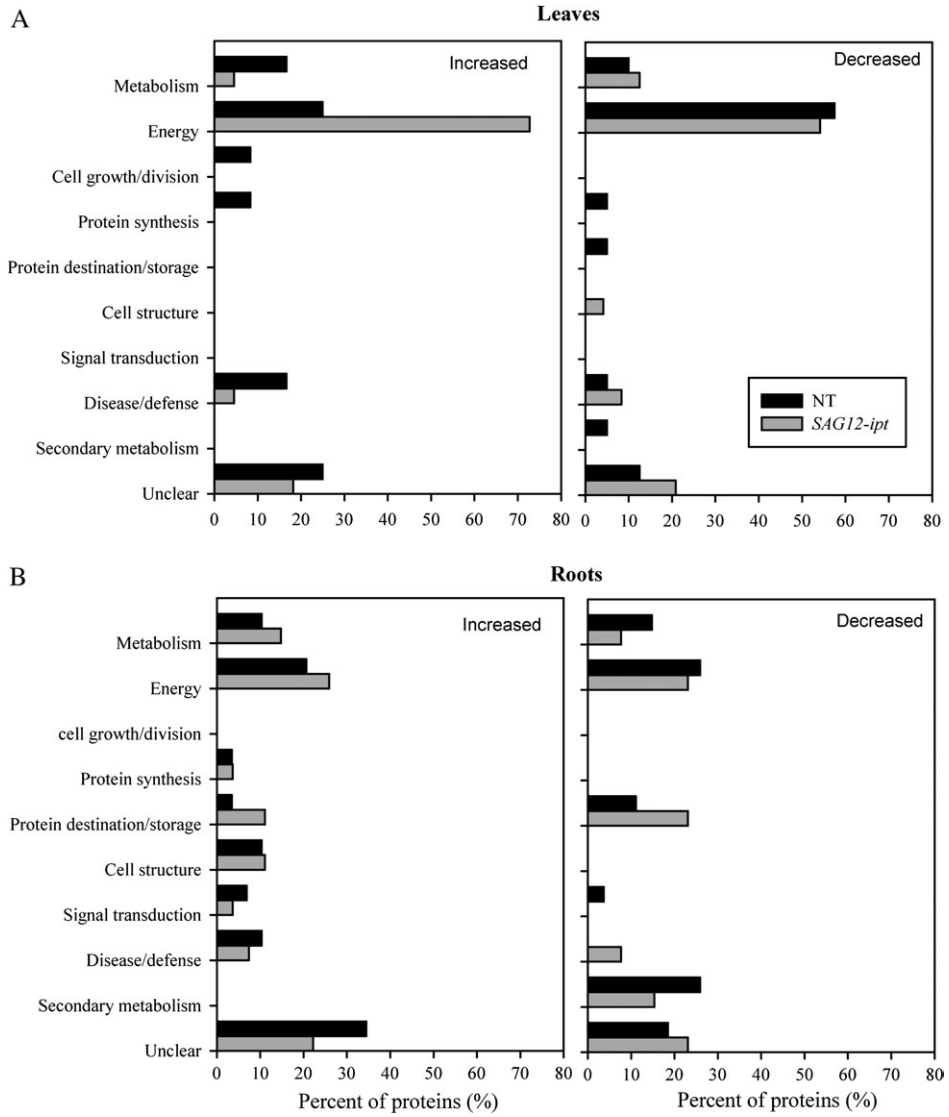

Fig. 7. Percentages of proteins exhibiting significant differential expression ($P \leqslant 0.05$) due to transgene expression or drought stress of *ipt* transgenic creeping bentgrass (*SAG12-ipt*) compared with null transformant (NT) lines within each functional category for (A) leaves and (B) roots.

are up-regulated by biotic stress (Shoresh and Harman, 2008). Their role in abiotic stress is unclear; however, in reference to its functions, increased levels of plasmodesmata proteins in *SAG12-ipt* plants could lead to enhanced root water transport properties. Root cell structure proteins such as actins (root R46, R47) were up-regulated in response to drought in both plant types, but R476 was increased more in *SAG12-ipt* roots. The abundance of β-5 tubulin protein (Y186) increased in response to water stress only in *SAG12-ipt* roots. Transcription of actin and tubulin structural proteins is highly hormonally regulated, and their accumulation levels affect cell growth, size, and cellular signalling under both non-stress and stressed conditions (Lang-Pauluzzi and Gunning, 2000; Klyachko, 2003). Tubulin proteins are regulated by osmotic stress (Komis *et al.*, 2002) and drought stress (Bagniewska-Zadworna, 2008), and are differentially regulated in *ipt* bentgrass in response to heat stress (Y Xu *et al.*, 2010). Maintenance of cell structural proteins may also be related to root viability (Klyachko, 2003; Bagniewska-Zadworna, 2008). Cell structural protein changes in *SAG12-ipt* roots could be a response to the influence of the *ipt* gene on root hormonal responses to drought, such as the ABA:CK ratio. Previously reported root hormonal changes in *SAG12-ipt* plants, the promotion of root growth (Merewitz *et al.*, 2010), and the changes in cell structural proteins suggest that *SAG12-ipt* plants have a root hormonal status conducive to increase cell structural integrity that stimulates root growth under drought stress conditions.

Protein changes in the signal transduction category

In roots, the abundance of three forms of GTP-binding proteins (root 29, 30, 31) was greater in NT plants under water stress relative to the well-watered control conditions and compared with *SAG12-ipt* plants under water stress.

GTP-binding proteins are responsible for the regulation of G proteins, which control many different cellular processes including cell division (Jones and Assmann, 2004). The difference in G protein accumulation could be related to the differential CK and ABA content in roots of *SAG12-ipt* and NT plants, as reported in Merewitz *et al.* (2010). The mechanism and function of several G proteins have not been fully elucidated, but they may play a role in guard cell responses to ABA and drought (Assmann, 2002; Perfus-Barbeouch, *et al.*, 2004). Thus, the role of GTP-binding proteins in NT plant responses to water stress is unclear, but increased activation or inactivation of G proteins by GTP-binding proteins could be related to stress damage and ABA in NT plants. The abundance of 14-3-3E was reduced by water stress in NT roots, whereas in *SAG12-ipt* roots it was increased. The 14-3-3E proteins are involved in signal transduction processes such as those that regulate cell elongation (Z Zhang *et al.*, 2010) and are associated with enzymes involved in primary metabolism, many of which were increased in *SAG12-ipt* roots relative to NT roots under stress, such as nitrate reductase (NR), sucrose synthase, GS, and GADPH (Roberts *et al.*, 2002). Thus, the maintenance of adequate levels of 14-3-3E protein may be a factor contributing to maintenance of signalling capabilities under water stress in *SAG12-ipt* plants.

Changes in proteins related to stress defence

The abundance of several antioxidant enzymes and chaperone proteins was altered by water stress or the expression of the *SAG12-ipt* gene. The abundance of 2-Cys peroxiredoxin (2-CP; leaf L11) decreased significantly in response to water stress in NT plants, but did not change in *SAG12-ipt* plants. 2-CP is an antioxidant enzyme that detoxifies hydroperoxides and peroxidized lipids; it plays an important role in the protection of the photosynthetic machinery, particularly PSII (Baier and Dietz, 1999), and may be directly regulated by CKs (Rhee *et al.*, 2005). An increase in 2-CP content was found in *Arabidopsis* in response to elevated CKs (Lochmanova *et al.*, 2008), and tall fescue plants overexpressing 2-CP exhibited increased stress tolerance (Kim *et al.*, 2010). Mitochondrial root 2-CP is essential for root growth under stress in *Arabidopsis* (Dietz *et al.*, 2006). The maintenance of 2-CP and other antioxidants within *SAG12-ipt* plants could contribute to better physiological performance under water stress, as demonstrated by the lower EL and MDA content at the same level of international water deficit (47% RWC) relative to NT plants.

CAT is an antioxidant enzyme that converts harmful H_2O_2 into H_2O and O_2, and CAT levels decrease during leaf senescence (Dhindsa *et al.*, 1981). Under well-watered conditions, *SAG12-ipt* leaves accumulated a greater protein content of two isoforms of CAT (leaf 111, L23) and total activity (based on total protein content) relative to NT lines, suggesting that CAT may be involved in *SAG12-ipt* inhibition of natural leaf senescence. Under drought stress, CAT activity in *SAG12-ipt* plants was generally increased followed by a slight decline by 5% SWC, which was reflected in differential responses of accumulation of the CAT isoforms by two-dimensional PAGE. Greater CAT content of some isoforms and overall activity, together with the delay in decline of F_v/F_m, suggested that *ipt* plants exhibited a reduction in drought-induced leaf senescence. This is consistent with previous research indicating a negative correlation of leaf senescence and CAT activity under optimal growth conditions (Dhindsa *et al.*, 1981), and in *ipt* tobacco, CAT was up-regulated by drought stress to a greater extent than in non-transgenic plants, and CAT remained more active for a greater duration of drought stress (Rivero *et al.*, 2007).

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

Biochemical assays (Leshem *et al.*, 1979; Pauls and Thomson, 1982) and exogenous application of ZR to heat-stressed bentgrass (Liu and Huang, 2002) have implicated that CKs may play an indirect role in the maintenance of antioxidant systems. It is largely accepted that the relationships of CKs to antioxidant systems is due to the role of CKs in cellular signalling, which leads to inhibition of senescence-promoting enzymes such as lipoxygenases (Brathe *et al.*, 2002) to slow the production of ROS caused by anabolic processes during senescence or stress. In addition, the results of MDA analysis are consistent with the potential that CKs may enhance the antioxidant system, resulting in a reduction of lipid damage by ROS, which has also been found in other *ipt* plant species (Qi-xian *et al.*, 2007). In *Pssu-ipt* tobacco, increased activity of PODs was also found under both non-stressed and drought stress conditions and was attributed to differences in peroxisome content between the wild-type and *ipt* plants (Synkova and Valcke, 2001). A strong antioxidant system under stress conditions plays a major role in stress tolerance of both leaves and roots of grass species (DaCosta and Huang, 2007; Wang and Jiang, 2007)

Summary

This study compared proteins expressed differentially in *SAG12-ipt* transgenic bentgrass and NT plants subjected to

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

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

References

Assmann SM. 2002. Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. *The Plant Cell* **14,** S355–S37.

Baek D, Jin Y, Jeong JC, *et al*. 2008. Suppression of reactive oxygen species by glyceraldehyde-3-phosphate dehydrogenase. *Phytochemistry* **69,** 333–338.

Bagniewska-Zadworna A. 2008. The root microtubule cytoskeleton and cell cycle analysis through desiccation of *Brassica napus* seedlings. *Protoplasma* **233,** 177–185.

Baier M, Dietz KJ. 1999. Protective function of chloroplast 2-cysteine peroxiredoxin in photosynthesis. Evidence from transgenic Arabidopsis. *Plant Physiology* **119,** 1407–1414.

Barrs HD, Weatherley PE. 1962. A re-examination of the relative turgidity techniques for estimating water deficits in leaves. *Australian Journal of Biological Sciences* **15,** 413–428.

Bauer D, Biehler K, Fock H, Carrayol E, Hirel B, Migge A, Becker TW. 1997. A role for cytosolic glutamine synthetase in the remobilization of leaf nitrogen during water stress in tomato. *Physiologia Plantarum* **99,** 241–248.

Bevan M, Bancroft I, Bent E, *et al*. 1998. Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of Arabidopsis thaliana. *Nature* **391,** 485–488.

Blum A. 1981. Cell membrane stability as a measure of drought and heat tolerance in wheat. *Crop Science* **21,** 43–47.

Bohnert HJ, Jensen RG. 1996. Strategies for engineering water-stress tolerance in plants. *Trends in Biotechnology* **14,** 89–97.

Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Brathe A, Andresen G, Gundersen LL, Malterud KE, Rise F. 2002. Antioxidant activity of synthetic cytokinin analogues: 6-alkynyl- and 6-alkenylpurines as novel 15-lipoxygenase inhibitors. *Bioorganic and Medicinal Chemistry* **10,** 1581–1586.

Braun HP, Schmitz UK. 1997. The mitochondrial processing peptidase. *International Journal of Biochemistry and Cell Biology* **29,** 1043–1045.

Bray EA. 1997. Plant responses to water deficit. *Trends in Plant Science* **2,** 48–54.

Caruso G, Cavaliere C, Foglia P, Gubbiotti R, Samperi R, Lagana A. 2009. Analysis of drought responsive proteins in wheat (*Triticum durum*) by 2D-PAGE and MALDI-TOF mass spectrometry. *Plant Science* **177,** 570–576.

Chance B, Maehly SK. 1955. Assay of catalase and peroxidases. *Methods in Enzymology* **2,** 764–775.

Chang W, Huang L, Shen M, Webster C, Burlingame AL, Roberts JKM. 2000. Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment and identification of proteins by mass spectrometry. *Plant Physiology* **122,** 295–317.

Chen M, Murata N. 2008. Glycine betaine: an effective protectant against abiotic stress in plants. *Trends in Plant Science* **13,** 499–505.

Chernyadev II. 2005. Effect of water stress on the photosynthetic apparatus of plants and the protective role of cytokinins: a review. *Applied Biochemistry and Microbiology* **41,** 115–128.

Chernyadev II. 2009. The protective action of cytokinins on the photosynthetic machinery and productivity of plants under stress. *Applied Biochemistry and Microbiology* **45,** 351–362.

Clark DG, Dervinis C, Barrett JE. 2004. Drought-induced leaf senescence and horticultural performance of transgenic *PSAG12-ipt* petunias. *Journal of the American Society of Horticultural Science* **129,** 93–99.

Corpas FJ, Barroso JB, Sandalio LM, Palma JM, Lupiáñez JA, del Río LA. 1999. Peroxisomal NADP-dependent isocitrate dehydrogenase. Characterization and activity regulation during natural senescence. *Plant Physiology* **121,** 921–928.

DaCosta M, Huang B. 2007. Changes in antioxidant enzyme activities and lipid peroxidation for bentgrass species in response to drought. *Journal of the American Society of Horticultural Science* **132,** 319–326.

Davies PJ. 2010. The plant hormones: their nature, occurrence, and functions. *Plant Hormones* A, 1–15.

Dhindsa RS, Plumb-Dhindsa P, Thorpe T. 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *Journal of Experimental Botany* **32,** 93–101.

Dietz KJ, Jacob S, Oelze ML, Laxa M, Tognetti V, Nunes de Miranda SM, Baier M, Finkemeier I. 2006. The function of peroxiredoxins in plant organelle redox metabolism. *Journal of Experimental Botany* **57,** 1697–1709.

Dietz KJ, Sauter A, Wichert K, Messdaghi D, Hartung W. 2000. Extracellular β-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. *Journal of Experimental Botany* **51,** 937–944.

Downs CG, Somerfield SD, Davey MC. 1997. Cytokinin treatment delays senescence but not sucrose loss in harvested broccoli. *Postharvest Biology and Technology* **11,** 93–100.

Ferreira S, Hjerno K, Larsen M, Wingsle G, Larsen P, Fey S, Roepstorff P, Pais MS. 2006. Proteome profiling of *Populus euphratica* Oliv. upon heat stress. *Annals of Botany* **98,** 361–377.

Foyer CH, Valadier MH, Migge A, Becker TW. 1998. Drought-induced effects on nitrate reductase activity and mRNA and on the coordination of nitrogen and carbon metabolism in maize leaves. *Plant Physiology* **117,** 283–292.

Georgopoulos C, Welch WJ. 1993. Role of the major heat shock proteins as molecular chaperones. *Annual Review of Cell Biology* **9,** 601–634.

Good AG, Zaplachinski ST. 1994. The effects of drought stress on free amino acid accumulation and protein synthesis in. *Brassica napus. Physiologia Plantarum* **90,** 9–14.

Heath RL, Packer L. 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* **125,** 189–198.

Heintz D, Erxleben A, High AA, Wurtz V, Reski R, Van Dorsselaer A, Sarnighausen E. 2006. Rapid alteration of the phosphoproteome in the moss *Physcomitrella patens* after cytokinin treatment. *Journal of Proteome Research* **5,** 2283–2293.

Hoagland DR, Arnon DI. 1950. The water culture method for growing plants without soil. *California Agricultural Experiment Station Circular* **347,** 1–32.

Hu Y, Jia W, Wang J, Zhang Y, Yang L, Lin Z. 2005. Transgenic tall fescue containing the *A. tumefaciens* ipt gene shows enhanced cold tolerance. *Plant Cell Reports* **23,** 705–709.

Ingram J, Bartels D. 1996. The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47,** 377–403.

Jones AM, Assmann SM. 2004. Plants: the latest model system for G-protein research. *EMBO Reports* **5,** 572–578.

Kalamaki MS, Alexandrou D, Lazari D, Merkouropoulos G, Fotopoulos V, Pateraki I, Aggelis A, Carrillo-Lopez A, Rubio-Cabetas MJ, Kanellis AK. 2009. Over-expression of a tomato *N*-acetyl-l-glutamate synthase gene (SlNAGS1) in *Arabidopsis thaliana* results in high ornithine levels and increased tolerance in salt and drought stresses. *Journal of Experimental Botany* **60,** 1859–1871.

Kim KH, Alam I, Lee KW, Sharmin SA, Kwak SS, Lee SY, Lee BH. 2010. Enhanced tolerance of transgenic tall fescue plants overexpressing 2-Cys peroxiredoxin against methyl viologen and heat stresses. *Biotechnology Letters* **2,** 571–576.

Klyachko NL. 2003. Phytohormones and cytoskeleton. *Russian Journal of Plant Physiology* **50,** 426–430.

Kocsy G, Laurie R, Szalai G, Szilágyi V, Simon-Sarkadi L, Galiba G, De Ronde JA. 2005. Genetic manipulation of proline levels affects antioxidants in soybean subjected to simultaneous drought and heat stresses. *Physiologia Plantarum* **124,** 227–235.

Koichi S, Hanagata N, Dubinsky Z, Baba S, Karube I. 2000. Molecular characterization of cDNA encoding oxygen evolving enhancer protein 1 increased by salt treatment in the mangrove. *Bruguiera gymnorrhiza. Plant and Cell Physiology* **41,** 1279–1285.

Komis G, Apostolakos P, Galatis B. 2002. Hyperosmotic stress induces formation of tubulin macrotubules in root-tip cells of Triticum turgidum: their probable involvement in protoplast volume control. *Plant and Cell Physiology* **43,** 911–922.

Konishi H, Kitano H, Komatsu S. 2005. Identification of rice root proteins regulated by gibberellin using proteome analysis. *Plant, Cell and Environment* **28,** 328–339.

Kruger NJ, von Schaewen A. 2003. The oxidative pentose phosphate pathway: structure and organisation. *Current Opinion in Plant Biology* **6,** 236–246.

Kudoyarova GR, Vysotskaya LB, Cherkozyanova A, Dodd IC. 2006. Effects of partial rootzone drying on the concentration of zeatin-type cytokinins in tomato (*Solanum lycopericum* L.) xylem sap and leaves. *Journal of Experimental Botany* **58,** 161–168.

Kulaeva ON, Burkhanova EA, Karavaiko NN, Selivankina SY, Porfirova SA, Maslova GG, Zemlyachenko YV, Börner T. 2002. Chloroplasts affect the leaf response to cytokinin. *Journal of Plant Physiology* **159,** 1309–1316.

Kusnetov VV, Oelmuller R, Sarwat MI, Porfirova SA, Cherepneva GN, Herrmann RG, Kulaeva ON. 1994. Cytokinins, abscisic acid and light affect accumulation of chloroplast proteins in *Lupinus luteus* cotyledons with notable effect on steady-state mRNA levels. *Planta* **194,** 318–327.

Lang-Pauluzzi I, Gunning BES. 2000. A plasmolytic cycle: the fate of cytoskeletal elements. *Protoplasma* **212,** 174–185.

Leshem YY, Grossman S, Frimer J, Ziv J. 1979. Endogenous lipoxygenase control and lipid associated free radical scavenging as modes of cytokinin action in plant senescence retardation. In: Appelqvist LA, Liljenberg C, eds. *Advances in the biochemistry and physiology of plant lipids*. Amsterdam, The Netherlands: Elsevier/ North-Holland Biomedical Press, 193–198.

Limami A, Phillipson B, Ameziane R, Pernollet N, Jiang A, Roy R, Deleens E, Chaumont-Bonnet M, Gressho PM, Hirel B. 1999. Does root glutamine synthetase control plant biomass production in *Lotus japonicus* L.? *Planta* **209,** 495–502.

Lipman DJ, Pearson WR. 1985. Rapid and sensitive protein similarity searches. *Science* **227,** 1435–41.

Liu X, Huang B. 2002. Cytokinin effects on creeping bentgrass response to heat stress. *Crop Science* **42,** 466–472.

Lochmanová G, Zdráhal Z, Konečná H, Koukalová S, Malbeck J, Souček P, Válková M, Nagavalli S, Kiran , Brzobohatý B. 2008. Cytokinin-induced photomorphogenesis in dark-grown Arabidopsis: a proteomic analysis. *Journal of Experimental Botany* **59,** 3705–3719.

McCabe MS, Garratt LC, Schepers F, Jordi WJRM, Stoopen GM, Davelaar E, Hans J, van Rhijn A, Power JB, Davey MR. 2001. Effects of PSAG12-IPT gene expression on development and senescence in transgenic lettuce. *Plant Physiology* **127,** 505–516.

Medford JI, Horgan R, Sawi ZE, Klee HJ. 1989. Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. *The Plant Cell* **1,** 403–413.

Merewitz E, Gianfagna T, Huang B. 2010. Effects of *SAG12-ipt* and *HSP18.2-ipt*. expression on cytokinin production, root growth and leaf senescence in creeping bentgrass exposed to drought stress. *Journal of the American Society for Horticultural Science* **135,** 230–239.

Merewitz E, Gianfagna T, Huang B. 2011. Photosynthesis, water use, and root viability under water stress as affected by expression of *SAG12-ipt* controlling cytokinin synthesis in *Agrostis stolonifera. Journal of Experimental Botany* **62,** 383–395.

Mocibob M, Ivic N, Bilokapic S, Maier T, Luic M, Ban N, Weygand-Durasevic I. 2010. Homologs of aminoacyl-tRNA synthetases acylate carrier proteins and provide a link between ribosomal and nonribosomal peptide synthesis. *Proceedings of the National Academy of Sciences, USA* **107,** 14585–14590.

Narita Y, Taguchi H, Nakamura T, Ueda A, Shi W, Takabe T. 2004. Characterization of the salt-inducible methionine synthase from barley leaves. *Plant Science* **167,** 1009–1016.

Patrick Jr. WH, Wyatt R. 1964. Soil nitrogen loss as a result of alternate submergence and drying. *Proceedings of the Soil Science Society of America* **28,** 647–653.

Pauls KP, Thompson JE. 1982. Effects of cytokinins and antioxidants on the susceptibility of membranes to ozone damage. *Plant and Cell Physiology* **23,** 821–832.

Perfus-Barbeoch L, Jones AM, Assmann SM. 2004. Plant heterotrimeric G protein function: insights from Arabidopsis and rice mutants. *Current Opinion in Plant Biology* **7,** 719–731.

Persson BC, Esberg B, Olafsson O, Björk GR. 1994. Synthesis and function of isopentenyl adenosine derivatives in tRNA. *Biochimie* **76,** 1152–60.

Popova LP, Tsonev TD, Lazova GN, Stoinova ZG. 1996. Drought- and ABA-induced changes in photosynthesis of barley plants. *Physiologia Plantarum* **96,** 623–629.

Qi-xian L, Zhi-yi B, Zhu-jun Z, Qiong-qiu Q, Bi-zeng M. 2007. Effects of osmotic stress on antioxidant enzymes activities in leaf discs of PSAG12-IPT modified gerbera. *Journal of Zhejiang University Science B* **8,** 458–464.

Rhee SG, Chae HZ, Kim K. 2005. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radical Biology and Medicine* **38,** 1543–1552.

Riccardi F, Gazeau P, de Vienne D, Zivy M. 1998. Protein changes in response to progressive water deficit in maize: quantitative variations and identification. *Plant Physiology* **117,** 1253–1263.

Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, Blumwald E. 2007. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. *Proceedings of the National Academy of Sciences, USA* **104,** 19631–19636.

Rivero RM, Shulaev V, Blumwald E. 2009. Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. *Plant Physiology* **150,** 1530–1540.

Roberts MR, Salinas J, Collinge DB. 2002. 14-3-3 proteins and the response to abiotic and biotic stress. *Plant Molecular Biology* **50,** 1031–1039.

Robson PRH, Donnison IS, Wang K, Frame B, Pegg SE, Thomas A, Thomas H. 2004. Leaf senescence is delayed in maize expressing the *Agrobacterium* IPT gene under the control of a novel maize senescence-enhanced promoter. *Plant Biotechnology Journal* **2,** 101–112.

Salekdeh GH, Siopongco J, Wade LJ, Ghareyazie B, Bennett J. 2002. *a*. A proteomic approach to analyzing drought- and salt-responsiveness in rice. *Field Crops Research* **76,** 199–219.

Salekdeh GH, Siopongco J, Wade LJ, Ghareyazie B, Bennett J. 2002. *b*. Proteomic analysis of rice leaves during drought stress and recovery. *Proteomics* **2,** 1131–1145.

Shoresh M, Harman GE. 2008. The molecular basis of shoot responses of maize seedlings to *Trichoderma harzianum* T22 inoculation of the root: a proteomic approach. *Plant Physiology* **147,** 2147.

Synkova H, Valcke R. 2001. Response to mild water stress in transgenic *Pssu-ipt* tobacco. *Physiologia Plantarum* **112,** 513–523.

Taylor NL, Heazlewood JL, Day DA, Millar AH. 2005. Differential impact of environmental stresses on the pea mitochondrial proteome. *Molecular and Cellular Proteomics* **4,** 1122–1133.

Topp GC, Davis JL, Annan AP. 1980. Electromagnetic determination of soil water content: measurements in coaxial transmission lines. *Water Resources Research* **16,** 574–582.

Turgeon AJ. 2008. *Turfgrass management*, 8th edn. Upper Saddle River, NJ: Pearson Prentice Hall.

UniProt Consortium. 2011. Ongoing and future developments at the universal protein resource. *Nucleic Acids Research* **39,** D214–D219.

Velasco R, Salamini F, Bartels D. 1994. Dehydration and ABA increase mRNA levels and enzyme activity of cytosolic GAPDH in the resurrection plant. *Craterostigma plantagineum. Plant Molecular Biology* **26,** 541–546.

Wang K, Jiang Y. 2007. Antioxidant responses of creeping bentgrass roots to waterlogging. *Crop Science* **47,** 232–238.

Xing J, Xu Y, Tian J, Gianfagna T, Huang B. 2010. Suppression of shade or heat-induced leaf senescence in creeping bentgrass through transformation with the ipt gene for cytokinin synthesis. *Journal of the American Society for Horticulture Science* **134,** 602–609.

Xu C, Huang B. 2010. Comparative analysis of drought responsive proteins in Kentucky bluegrass cultivars contrasting in drought tolerance. *Crop Science* **50,** 2543–2552.

Xu C, Sibicky T, Huang B. 2010. Protein profile analysis of salt-responsive proteins in leaves and roots in two cultivars of creeping bentgrass differing in salinity tolerance. *Plant Cell Reports* **29,** 595–615.

Xu C, Xu Y, Huang B. 2008. Protein extraction for 2-dimensional gel electrophoresis of proteomic profiling in turfgrass. *Crop Science* **48,** 1608–1614.

Xu Q, Huang B. 2004. Antioxidant metabolism associated with summer leaf senescence and turf quality decline for creeping bentgrass. *Crop Science* **44,** 553–560.

Xu Y, Gianfagna T, Huang B. 2010. Proteomic changes associated with expression of a gene (*ipt*) controlling cytokinin synthesis for improving heat tolerance in a perennial grass species. *Journal of Experimental Botany* **61,** 3273–3289.

Xu Y, Tian J, Gianfagna T, Huang B. 2009. Effects of *SAG12-ipt* expression on cytokinin production, growth and senescence of creeping bentgrass (*A. stolonifera* L.) under heat stress. *Plant Growth Regulation* **57,** 281–291.

Yang Y, Kwon HB, Peng HP, Shih MC. 1993. Stress responses and metabolic regulation of glyceraldehyde-3-phosphate dehydrogenase genes in. *Arabidopsis. Plant Physiology* **101,** 209–216.

Yang J, Zhang J, Wang Z, Zhu Q, Liu L. 2002. Abscisic acid and cytokinins in the root exudates and leaves and their relationship to senescence and remobilization of carbon reserves in rice subjected to water stress during grain filling. *Planta* **215,** 645–652.

Zhang J, Kirkham MB. 1996. Antioxidant responses to drought in sunflower and sorghum seedlings. *New Phytologist* **132,** 361–373.

Zhang J, Van Toai T, Huynh L, Preiszner J. 2000. Development of flooding-tolerant arabidposis by autoregulated cytokinin production. *Molecular Breeding* **6,** 135–144.

Zhang P, Wang WQ, Zhang GL, Kaminek M, Dobrev P, Xu J, Gruissem W. 2010. Senescence-inducible expression of isopentenyl transferase extends leaf life, increases drought stress resistance and alters cytokinin metabolism in cassava. *Journal of Integrative Plant Biology* **52,** 653–669.

Zhang ZT, Zhou Y, Li Y, Shao SQ, Li BY, Shi Y, Li XB. 2010. Interactome analysis of the six cotton 14-3-3s that are preferentially expressed in fibres and involved in cell elongation. *Journal of Experimental Botany* **61,** 3331–3344.

Zhao Y, Du H, Wang Z, Huang B. 2011. Identification of proteins associated with water-deficit tolerance in C4 perennial grass species. *Cynodon dactylon* × *Cynodon transvaalensis and Cynodon dactylon. Physiologia Plantarum* **141,** 40–55.