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

Transgenic modification of plants to incorporate the ipt gene encoding an enzyme in the CK biosynthesis pathway, adenine isopentenyl transferase, increases endogenous CK content, resulting in improved drought tolerance in various plant species (Clark et al., 2004; Rivero et al., 2007; Merewitz et al., 2010, 2011; P Zhang et al., 2010). Merewitz et al. (2010, 2011) reported that compared with null transformant (NT) control plants, creeping bentgrass (a C3 perennial grass species) containing the ipt gene under a senescence-activated promoter (SAG12-ipt) exhibited higher photosynthesis rates, photochemical efficiency (Fv/Fm), 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 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 coordinate 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: \( \frac{FW - DW}{FW} \times 100 \) (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 \( \frac{C_i}{C_{max}} \times 100 \) (Blum, 1981).

Leaf photochemical efficiency (\( \frac{F_v}{F_m} \)) was evaluated as the ratio of the variable fluorescence (\( F_v \)) to the maximal fluorescence (\( F_m \)). The value was 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 \( \frac{F_v}{F_m} \) 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 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 \( A_{532} \) 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 0.5–1.0 g of leaf or 1 g of root sample 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 (V.1.9, 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 (totaling 40 plants of each plant type). The effects of watering treatment, plant materials, and corresponding interactions were determined by analysis of variance according to the general linear model procedure of SAS (version 9.0; SAS Institute, Cary, NC, USA). Differences between watering treatments and plant means were separated by Fisher’s protected least significance difference (LSD) test at the 0.05 probability level.

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

Leaf physiological responses to water stress
Grass quality, EL, RWC, and $F_v/F_m$ were not significantly different between the NT and SAG12-ipt plants under well-watered conditions or at the initiation of water stress treatment when SWC was maintained at 25% (Fig. 2). Leaf colour and turgidity estimated as grass quality of both NT and SAG12-ipt decreased in response to water stress, but at a slower rate of decline, and were maintained at a significantly higher level for SAG12-ipt plants than the NT plants (Fig. 2A). At the end of the water stress treatment when the SWC declined to 5%, quality ratings were maintained at 4 for SAG12-ipt and only 1 for NT plants. Leaf EL, RWC, and $F_v/F_m$ also declined in response to water stress in both the NT and SAG12-ipt plants (Fig. 2C, D). Leaf $F_v/F_m$ declined when SWC dropped to 5% and was significantly higher in SAG12-ipt (0.7) than in NT plants (0.5). The decline of RWC and the corresponding increase in EL occurred at a higher SWC for NT relative to SAG12-ipt plants. Leaf RWC dropped to ~7% when SWC reached 10% for NT and 5% for SAG12-ipt.

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

Root physiological responses to drought stress
Root: shoot ratios increased with decreasing SWC in both NT and SAG12-ipt plants, but the ratios were significantly greater in the SAG12-ipt plants at 10% and 5% SWC (Fig. 4A). Root MDA content increased with declining SWC from 25% to 5% in NT plants, but the increases in root MDA content did not occur until SWC decreased to 5% in SAG12-ipt plants (Fig. 4B). At an SWC between 25%
and 10%, the NT roots had significantly higher MDA content than those of the SAG12-ipt plants. Root SOD activity decreased during the decline in SWC from 25% to 5% in both NT and SAG12-ipt plants, but the SAG12-ipt plants had significantly greater SOD activity in roots than the NT roots at 25, 10, and 5% SWC (Fig. 4C). Root POD activity was relatively unchanged by decreasing SWC and was not significantly different between SAG12-ipt and NT roots at an SWC between 25% and 10%. At 5% SWC, POD activity was significantly higher in NT roots. No difference in CAT activity was detectable in roots of both NT and SAG12-ipt plants (data not shown).

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

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

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

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

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

Specific proteins responsive to SAG12-ipt expression under water stress

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

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

Discussion

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

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

The water depletion rate, as indicated by changes in SWC content (Fig. 1) during water stress, was similar...
between the NT and SAG12-ipt plants, but at the same level of SWC and RWC (i.e. 47% RWC), transgenic plants maintained lower EL and MDA content. These data suggested that the improved shoot and root growth in the SAG12-ipt plants under water stress was not related to avoidance mechanisms such as a differential water depletion rate, but rather could be due to enhanced tolerance mechanisms, which is supported by the analysis of antioxidant enzyme activities. Leaf SOD, CAT, and POD activity and root SOD activity were significantly higher in SAG12-ipt plants compared with those in the NT plants. The tolerance mechanism most directly affected by the ipt gene may be increased antioxidant activity, rather than the direct regulation of water loss and cellular dehydration.

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 ≤0.05).
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 watered Sag12-ipt from NT) |
|-----|--------------|---------------------------------------------------------------------------------|---------------------------------------------|---------------------------------------------------------------------------------|
|     |              | NT                  | SAG12-ipt                           | NT                  | SAG12-ipt                           |
| 3   | Glycine decarboxylase P subunit/Victorin binding protein [A. thaliana] | ns                  | 59.5 ns                            | ns                  | ns                             |
| 4   | Alanine aminotransferase [A. thaliana] | ns                  | ns ns                            | ns                  | ns                             |
| 6   | Aminomethyltransferase [O.sativa ('japonica' cultivar-group)] | ns                  | 47.3 ns                            | ns                  | ns                             |
| 9   | Methionine synthase [MS, Hordeum vulgare subsp. vulgare] | ns                  | -30.9 ns                           | ns                  | ns                             |
| 10  | Methionine synthase [MS, H. vulgare subsp. vulgare] | ns                  | -27.1 89.9* ns                    | ns                  | ns                             |
| 11  | Aspartate aminotransferase [O. sativa] | ns                  | ns ns                            | ns                  | ns                             |
| 12  | Cell wall beta-glucosidase ([α-D-glucan exohydrolase) [Secale cereale] | ns                  | ns ns                            | ns                  | ns                             |
| 13  | Cell wall beta-glucosidase ([β-D-glucan exohydrolase) [Triticum aestivum] | ns                  | -20.2 ns                           | ns                  | -38.7 ns                        |
| 15  | UDP-sulfoquinovose synthase [O. sativa ('japonica' group)] | ns                  | ns -78.1 ns                       | ns                  | 34.6 ns                        |
| 16  | Adenosine diphosphate glucose pyrophosphatase [H. vulgare subsp. vulgare] | ns                  | -41.4 ns                           | ns                  | 71.7 ns                        |
| L1  | Possible: 3-hydroxy-3-methylglutaryl coenzyme A reductase [Malus x domestica] (40 kD, pl 8.0, gi|71199371) | ns                  | ns ns                            | ns                  | 29.7 ns                        |
| L24 | Possible: Aspartate aminotransferase [Pinus pinaster] (53 kD, pl 7.5, gi|59932915) | ns                  | -45.4 ns                           | ns                  | ns                             |
| 110 | Glycolate oxidase | ns                  | ns -43.5 ns                       | ns                  | ns                             |
| 18  | RuBisCO large subunit [Psathyrostachys fragilis subsp. Fragilis] | ns                  | -20.2 45.2* ns                    | ns                  | ns                             |
| 20  | RuBisCO large subunit [Bulbine succulenta] | ns                  | -30.9 ns                           | ns                  | ns                             |
| 26  | RuBisCO large subunit [Aira praecox] | ns                  | ns 34.2 ns                        | ns                  | ns                             |
| 28  | RuBisCO small subunit [Avena maroccana] | ns -24.6 ns           | ns ns                            | ns                  | ns                             |
| 29  | RuBisCO small subunit [T. aestivum] | ns                  | 35.0 ns                           | ns                  | ns                             |
| 30  | RuBisCO small subunit [T. aestivum] | ns                  | 50.6 ns                           | ns                  | ns                             |
| 32  | RuBisCO small subunit [A. maroccana] | ns                  | ns -16.1 ns                       | ns                  | ns                             |
| 34  | RuBisCO small subunit [Brorns catharticus] | ns                  | ns 19.2 ns                        | ns                  | ns                             |
| 36  | RuBisCO small subunit [T. aestivum] | ns                  | ns -30.5 ns                       | ns                  | ns                             |
| 38  | RuBisCO activase [Nicotiana tabacum] | ns                  | ns -20.1 ns                       | ns                  | 72.7 ns                        |
| 41  | RuBisCO activase 1 [Gossypium hirsutum] | ns                  | ns -67.8 ns                       | ns                  | ns                             |
| 42  | RuBisCO activase 1 [G. hirsutum] | ns                  | ns -32.0 ns                       | ns                  | ns                             |
| 44  | Phosphoribulokinase (Phosphopentokinase) [O. sativa ('japonica' group)] | ns                  | ns -9.9 33.1* ns                  | ns                  | ns                             |
| 11  | GAPDH B, chloroplast precursor [O. sativa ('japonica' group)] | ns                  | 117.0 ns                           | ns                  | ns                             |
| 45  | GAPDH, cytosolic [O. sativa ('japonica' group)] | ns                  | ns -28.5 21.2 ns                  | ns                  | ns                             |
| 49  | GAPDH B, chloroplast precursor [O. sativa ('japonica' group)] | ns                  | ns 49.8 ns                        | ns                  | ns                             |
| 51  | GAPDH A, chloroplast [O. sativa ('japonica group)] | ns                  | 31.8 -30.9 ns                     | ns                  | 26.0 ns                        |
| 54  | GAPDH, cytosolic [O. sativa ('japonica' group)] | ns                  | ns -37.4 48.3* ns                  | ns                  | ns                             |
| 55  | GAPDH A, chloroplast [O. sativa ('japonica group)] | ns                  | ns -33.9 ns                       | ns                  | ns                             |
| 56  | GAPDH A, chloroplast [O. sativa ('japonica group)] | ns                  | ns -60.1 ns                       | ns                  | ns                             |
| 57  | GAPDH A, chloroplast [O. sativa ('japonica group)] | ns                  | ns ns                            | ns                  | ns                             |
| 58  | GAPDH B, chloroplast precursor [O. sativa ('japonica group)] | ns                  | ns ns                            | ns                  | ns                             |
| 60  | Cytoplasmic fructose-biphosphate (FBP) aldolase [O.sativa] | ns                  | ns -32.1 ns                       | ns                  | -14.2 ns                       |
| 61  | Cytoplasmic aldolase [O. sativa] | ns                  | ns ns                            | ns                  | -27.8 ns                       |
| 63  | Chloroplastic aldolase [O. sativa] | ns                  | 23.5 ns                           | ns                  | ns                             |
| 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 watered Sag12-ipt from NT) |
|-----|--------------|--------------------------------------|------------------------------------------|---------------------------------------------|
| 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  | Hydroxyproline reductase [O. sativa ('japonica group')] | ns | ns | 22.7 | ns |
| 75  | ATPase, β subunit [H. vulgare] | ns | -30.6 | ns | ns |
| 76  | ATP synthase subunit II [O. sativa ('japonica group')] | -35.7 | ns | ns | ns |
| 77  | 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 | 67.4 | ns |
| 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, pl 8.1, gi544700) | ns | 48.6 | ns | ns |
| L14 | atp1 [T. aestivum] (55kD, pl 5.7, gi81176509) | ns | -36.7 | 25.3* | 47.3 |
| L15 | Isocitrate dehydrogenase [NADP], chloroplastic precursor (48kD, pl 6.2, gi2497259) | ns | -42.2 | -20.1* | ns |
| L18 | PSI type III chlorophyll a/b-binding protein (29kD, pl 8.6, gi430947) | ns | ns | 80.1 | ns |
| L19 | Triosephosphate isomerase, cytosolic; (27kD, pl 5.4, gi2507469) | ns | ns | -28.4 | ns |
| L2  | Possible: putative cytochrome c oxidase subunit IIP517 (2kD, pl 9.6, gi109892850) | ns | 76.7 | 91.8* | 18.7 |
| L20 | 6-phosphogluconate dehydrogenase, decarboxylating [Chlamydomonas reinhardtii] (61 kD, pl.8.4, gi15225026) | ns | -43.1 | ns | ns |
| L31 | Putative phosphogluconate dehydrogenase [O. sativa ('japonica group')] (45kD, pl5.4, gi55295906) | 13.2 | -20.2 | -20.7 | ns |
| L36 | Glyceraldehyde 3-phosphate dehydrogenase [A. thaliana] (43kD, pl5.6,gi336390) | 71.9 | ns | -39.3 | ns |
| L4  | Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (33kD, pl 6.2, gi120668) | ns | ns | 67.6 | ns |
| L6  | Possible: ferredoxin [Zea mays] (41kD, pl 8.7, gi162458489) | ns | -34.4 | ns | ns |
| L7  | Photosystem I subunit VII [Oryza sativa ('japonica group')] (8.9kD, pl6.5) | ns | ns | 147.5 | 19.6 |
| L9  | Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [Avena sativa] (18kD, pl 8.8, gi4038695) | 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, gi|109893655)
- **L5** Putative RNA binding protein - Arabidopsis thaliana (43kD, pi 8.2, gi|3850621)
- **L17** Possible: mitochondrial elongation factor Tu - Arabidopsis thaliana (52 kD, pi 5.5, gi|148671)
- **L30** Possible: Elongation factor G, chloroplast precursor - Ostreococcus tauri (86kD, pi 5.3, gi|16059008)

**Category 05 Protein synthesis**

- **120** Chloroplast translational elongation factor Tu - O. sativa (japonica' group) (50kD, pi 6.1)
- **L5** Putative RNA binding protein - Arabidopsis thaliana (43kD, pi 8.2, gi|3850621)
- **L17** Possible: mitochondrial elongation factor Tu - Arabidopsis thaliana (52 kD, pi 5.5, gi|148671)
- **L30** Possible: Elongation factor G, chloroplast precursor - Ostreococcus tauri (86kD, pi 5.3, gi|16059008)

**Category 06: Protein destination/storage**

- **101** Heat shock protein 70 - Cucumis sativus
- **103** RuBisCO large subunit-binding protein subunit beta, (60 kDa chaperonin subunit beta)
- **L3** Putative protein disulphide isomerase (PDI) - Brassica napus var. napus (26kD, pi 6.5, gi|45593261)
- **L34** OSJNBA0039C07.4 [HSP93 III] - O. sativa (japonica' group) (98kD, pi 5.8, gi|38347158)

**Category 09 Cell structure**

- **L35** Type IIIa membrane protein cp-wap13 - Vigna unguiculata (39kD, pi 6.2, gi|2218152)

**Category 11 Disease/defense**

- **109** Catalase-1 - O. sativa (japonica' group)
- **110** Catalase-1 - O. sativa (japonica' group)
- **111** Catalase-1 - O. sativa (japonica' group)
- **114** Ascorbate peroxidase APX4 - A. thaliana
- **115** Ascorbate peroxidase APX7, chloroplastic - O. sativa (japonica' group)
- **116** Glutathione-S-transferase (GST) - H. vulgare
- **L11** 2-Cys peroxiredoxin BAS1, chloroplast precursor - T. aestivum (23kD, pi 5.7, gi|2829687)
- **L23** Possible: catalase (56kD, pi 6.7, gi|1705626)
- **L37** Possible: ascorbate peroxidase - A. thaliana (28kD, pi 5.9, gi|555576)

**Category 20 Secondary metabolism**

- **L22** Glycine decarboxylase P subunit - Tritordeum sp. (111kD, pi 6.5, gi|2565305)
- **L27** S-adenosylmethionine synthetase (gi|3914019)

**Unclear**

- **111** Unknown
- **118** Unknown
- **119** Unknown
- **122** Unknown
- **123** Unknown
- **127** Unknown
- **131** Unknown
- **137** Unknown

Cytokinin regulation of proteins associated with drought tolerance | 5321

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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)

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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) NT | Differences between lines under drought (% change in drought treated Sag12-ipt from NT) |
|-----|--------------|---------------------------------------------------------------------------------|-------------------------------------------|--------------------------------------------------------------------------------------------------|
| 139 | Unknown      | ns                                                                               | -30.9 ns                                  | ns                                                                                               |
| 141 | Unknown      | ns                                                                               | ns                                        | 22.2 ns                                                                                  |
| 142 | Unknown      | ns                                                                               | 52.8 *                                    | 27.7 ns                                                                                  |
| 148 | Unknown      | ns                                                                               | ns                                        | -62.6 ns                                                                                  |
| L12 | Unknown      | ns                                                                               | -68.1                                    | 261.2 *                                                                                 |
| L26 | Unknown      | ns                                                                               | ns                                        | 33.1 ns                                                                                  |
| L32 | Unknown      | 77.1                                                                             | 47.0 *                                    | -36.3 ns                                                                                  |

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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 photosensitization and may increase endogenous H$_2$O$_2$ (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 chloroplastic 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 chloroplastic 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 chloroplastic 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 isozymes 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 | NT | SAG12-ipt |
| 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 |
| 72  | Cytosolic glutamine synthetase [Populus alba x Populus tremula] | 109.6 | ns | ns | 147.6* |
| 52  | Sucrose synthase (EC 2.4.1.13) Sts1 [H. vulgare] | ns | ns | 81.7* | 35.9 |
| 73  | Cytosolic GAPDH (phosphorylation) (EC 1.2.1.12) [H. vulgare] | 14.2 | 35.5 | ns | 72.4 |
| 74  | 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 |
| 76  | Isocitrate dehydrogenase [NADP], chloroplast precursor | 55.9 | -17.7 | ns | 17.8 |
| 77  | 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 |
| 84  | L-malate dehydrogenase (MDH) [A. thaliana] (42kD, pl 9.0, gi15232820) | ns | ns | -23.0 | ns |
| 85  | Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (33kD, pl 6.2, gi120668) | 30.8 | 40.6 | ns | ns |
| 86  | Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (33kD, pl 6.2, gi120668) | 17.1 | ns | ns | ns |
| 87  | NADPH producing dehydrogenase of the oxidative pentose phosphate pathway [Zea mays] (53kD, pl 5.9, gi16246282) | ns | -17.8 | ns | -38.7 |
| 88  | Cytosolic 6-phosphogluconate dehydrogenase [O. sativa] (52kD, pl 6.5, gi38426301) | ns | ns | -64.4 | ns |
| 89  | Ribulose-1,5-bisphosphate carboxylase, large subunit [Dolichos biflorus] (52kD, pl 6.5, gi1770216) | ns | 54.0 | 79.7* | ns |
| 90  | O-methyltransferase 4 [T. aestivum] (38kD, pl 5.6, gi145693798) | ns | 10.8 | ns | ns |
| 91  | Ferredoxin-NADP reductase precursor [Z. mays] (36kD, pl8.4) homologue | ns | 106.3 | 94.8 | ns |
| 92  | ATP synthase beta subunit [T. aestivum] (59kD, pl 4.7, gi525291) | ns | ns | 148.0 | 77.3 |
| 93  | Ferredoxin-nitrite reductase precursor [T. aestivum] (69kD, pl6.9, gi218963620) | -51.9 | -57.8 | ns | ns |
| 94  | Glucose-6-phosphate isomerase (GPI) cytosolic (62 kD, pl 6.96, Accn P49105) | ns | ns | ns | 34.7 |

Category 01 Metabolism

Category 02 Energy
| 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                   |                                              |
|      |                                                                             | -69.6                   | -50.5*                      | -18.0                                       |
| 57   | Putative asparagine-tRNA ligase (EC 6.1.1.22) [O. sativa]                    | 12.9                    | 52.2                        | 31.2                                        |
| 23   | Mitochondrial processing peptidase α-chain (MPP) (EC 3.4.24.64)              | ns                      | -52.7                      | -31.2                                       |
| 86   | Endoplasmic reticulum precursor (HSP90) [T. aestivum]                        | ns                      | 52.3                        | 51.0                                        |
| 88   | 70 kDa heat shock cognate [Vigna radiata]                                   | ns                      | 121.1                      | ns                                          |
| 90   | Protein disulfide isomerase (PDI) 3 precursor [T. aestivum]                  | -53.8                   | -16.4                      | ns                                          |
| R6   | Os09g0505600 [O. sativa ‘japonica’] (24kD, pl 6.4) (possible proteasome function) | ns                      | -29.7                      | ns                                          |
| R29  | Possible: Proteasome subunit α type-7 (28kD, pl8.4)                         | ns                      | 143.1                      | 153.6                                       |
| R40  | Possible: heat shock protein 93 [A. thaliana] (81kD, pl5.0)                 | ns                      | 35.5                        | 39.6                                        |
| Y153 | Putative t-complex protein 1 theta chain [O. sativa]                         | -69.0                   | 50.5                        | ns                                          |
|      |                                                                             |                         |                             |                                              |
| 59   | Ran (Small GTP-binding protein) [Ran2] [O. sativa]                           | ns                      | ns                          | -18.5                                       |
| 26   | Reversibly glycosylated polypeptide [T. aestivum]                            | ns                      | 52.4                        | 40.0                                        |
| R46  | Actin [Cleistogenes songonica] (42kD, pl5.5)                                 | ns                      | 77.0                        | 109.0*                                      |
| R47  | Actin-1 (42kD, pl 5.5)                                                      | ns                      | 99.4                        | 133.9*                                      |
| Y18  | β-5 tubulin [Triticum aestivum]                                             | ns                      | ns                          | 35.9                                        |
|      |                                                                             |                         |                             |                                              |
| 29   | GTP-binding protein [O. sativa]                                              | ns                      | 49.4                        | ns                                          |
| 30   | GTP-binding protein beta chain homolog curled-leaved [N. tabacum]            | ns                      | 43.2                        | ns                                          |
| 31   | GTP-binding protein beta chain [N. tabacum]                                 | ns                      | ns                          | -7.8                                        |
| R49  | Possible: 14-3-3E [H. vulgare subsp. vulgare] (29kD, pl4.8)                 | ns                      | -17.5                      | 31.2*                                       |
|      |                                                                             |                         |                             |                                              |
| 33   | Probable peroxidase (EC 1.11.1.1) 1 precursor anionic [Z. mays]             | ns                      | ns                          | -32.2                                       |
| 34   | Probable peroxidase (EC 1.11.1.1) 1 precursor anionic [Z. mays]             | ns                      | 27.9                        | ns                                          |
| 64   | Superoxide dismutase (EC 1.15.1.1) [Mn] 3.2 precursor [Z. mays]            | ns                      | 77.3                        | ns                                          |
| 66   | Slt (Stress inducible protein) [Glycine max]                                | ns                      | ns                          | -32.0                                       |
| R5   | Ascorbate peroxidase [H. vulgare subsp. vulgare] (27kD, pl 5.8)             | ns                      | 79.2                        | 46.2                                        |
|      |                                                                             |                         |                             | -24.3                                       |
| 38   | dDTP-glucose 4-6-dehydratases-like protein [A. thaliana]                    | ns                      | -16.7                       | ns                                          |
| 39   | Adenosylhomocysteinase (EC 3.3.1.1) [T. aestivum]                            | ns                      | -35.3*                      | -54.4                                       |
| 40   | S-adenosylmethionine synthase (SAM) (EC 2.5.1.6) [A. thaliana]              | ns                      | -31.7                       | -27.2                                       |
| 41   | SAMs (EC 2.5.1.6) [Dendrobaena crumenata]                                   | ns                      | -31.0                       | ns                                          |
| 68   | UDP-glucose 6-dehydrogenase (EC 1.1.1.22) [Glycine max]                    | ns                      | 38.3                        | ns                                          |
| 93   | UDP-glucose dehydrogenase [O. sativa ‘japonica’ group]                     | ns                      | -13.9                       | ns                                          |
| R18  | S-adenosylmethionine synthetase (43kD, pl 5.4)                              | ns                      | -30.0                       | ns                                          |
|      |                                                                             |                         |                             | 33.9                                        |
| 46   | Unknown                                                                    | ns                      | 51.7                        | ns                                          |
| 47   | Unknown                                                                    | ns                      | -11.7                       | ns                                          |
| 94   | Os04g0650800 [O. sativa ‘japonica’ group]                                   | ns                      | -31.5                       | ns                                          |
| 97   | Unknown                                                                    | ns                      | 34.2                        | ns                                          |
| 98   | Unknown                                                                    | ns                      | -3.1                        | -39.8*                                      |
| 99   | Unknown                                                                    | ns                      | 24.3                        | ns                                          |
| 101  | Unknown                                                                    | ns                      | 52.9                        | ns                                          |
| 102  | Unknown                                                                    | ns                      | 205.0                       | ns                                          |
| 104  | Unknown                                                                    | ns                      | 7.0*                        | -26.1                                       |
| R2   | Putative n40c1 protein - rice [O. sativa ‘japonica’ group] (42 kD, pl 6.2) | ns                      | 117.9                       | ns                                          |
| R16  | Unknown                                                                    | -61.8                   | -56.3                       | 50.1                                        |
| R19  | unknown                                                                    | ns                      | ns                          | 25.5                                        |
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.

### 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) |
|------|--------------|--------------------------------------------------------------------------------------|--------------------------------------------|----------------------------------------------------------------------------------|
| R28  | unknown      | ns                                                                                   | 180.8                                      | ns                                                                               |
| R30  | unknown      | ns                                                                                   | 74.3                                       | ns                                                                               |
| R31  | unknown      | ns                                                                                   | -3.2                                      | ns                                                                               |
| R32  | unknown      | ns                                                                                   | 115.8                                      | ns                                                                               |
| R33  | unknown      | ns                                                                                   | ns                                        | -43.6                                                                           |
| R34  | unknown      | ns                                                                                   | ns                                        | -43.6                                                                           |
| R48  | unknown      | ns                                                                                   | ns                                        | -23.0                                                                           |
| R51  | unknown      | ns                                                                                   | ns                                        | 14.5                                                                            |
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, iP, is directly associated with tRNAs in translation, and derivatives of iP 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 endoplasmic homologue precursor of HSP90 (root 86), Hsp70 cognate (root 88), and the proteasome subunit alpha type-7 (root R29), and a decrease in a mitochondrial processing peptidase (MPP; root 23). Increases in the abundance of these proteins were most pronounced in SAG12-ipt roots, whereas decreases were more prominent in NT roots. Both PDI and a ferredoxin-nitrite reductase precursor were lower in SAG12-ipt than in NT plants under well-watered conditions. The abundance of root Hsp83 (R40) was increased by drought in response to water stress in SAG12-ipt, but a significant change did not occur in NT. Thus, improvements in drought tolerance of SAG12-ipt relative to NT could be related to increased protein chaperone and import function capabilities since Hsp90, Hsp70, and PDI are all involved in assisting protein folding (Georgopoulos and Welch, 1993). Similarly, MPP is involved in protein import to (Braun and Schmitz, 1997) and replacement of damaged proteins in the mitochondria during stress conditions (Taylor et al., 2005).

Changes in proteins functioning within the cellular structure and growth category

One leaf protein in the cell structure category, a type IIIa membrane protein cp-wap13 (leaf L35), exhibited differential accumulation in response to water stress between the SAG12-ipt and NT plants. Cp-wap13 proteins are associated with the Golgi apparatus as well as cellulose biosynthesis in the cell wall, primarily in plasmodesmata, and...
are up-regulated by biotic stress (Shoresh and Harman, 2008). Their role in abiotic stress is unclear; however, in reference to its functions, increased levels of plasmodesmata proteins in SAG12-ipt plants could lead to enhanced root water transport properties. Root cell structure proteins such as actins (root R46, R47) were up-regulated in response to drought in both plant types, but R476 was increased more in SAG12-ipt roots. The abundance of β-5 tubulin protein (Y186) increased in response to water stress only in SAG12-ipt roots. Transcription of actin and tubulin structural proteins is highly hormonally regulated, and their accumulation levels affect cell growth, size, and cellular signalling under both non-stress and stressed conditions (Lang-Pauluzzi and Gunning, 2000; Klyachko, 2003). Tubulin proteins are regulated by osmotic stress (Komis et al., 2002) and drought stress (Bagniewska-Zadworna, 2008), and are differentially regulated in ipt bentgrass in response to heat stress (Y Xu et al., 2010). Maintenance of cell structural proteins may also be related to root viability (Klyachko, 2003; Bagniewska-Zadworna, 2008). Cell structural protein changes in SAG12-ipt roots could be a response to the influence of the ipt gene on root hormonal responses to drought, such as the ABA:CK ratio. Previously reported root hormonal changes in SAG12-ipt plants, the promotion of root growth (Merewitz et al., 2010), and the changes in cell structural proteins suggest that SAG12-ipt plants have a root hormonal status conducive to increase cell structural integrity that stimulates root growth under drought stress conditions.

Protein changes in the signal transduction category

In roots, the abundance of three forms of GTP-binding proteins (root 29, 30, 31) was greater in NT plants under water stress relative to the well-watered control conditions and compared with SAG12-ipt plants under water stress.
GTP-binding proteins are responsible for the regulation of G proteins, which control many different cellular processes including cell division (Jones and Assmann, 2004). The difference in G protein accumulation could be related to the differential CK and ABA content in roots of SAG12-ipt and NT plants, as reported in Merewitz et al. (2010). The mechanism and function of several G proteins have not been fully elucidated, but they may play a role in guard cell responses to ABA and drought (Assmann, 2002; Perfus-Barbeouch, et al., 2004). Thus, the role of GTP-binding proteins in NT plant responses to water stress is unclear, but increased activation or inactivation of G proteins by GTP-binding proteins could be related to stress damage and ABA in NT plants. The abundance of 14-3-3E was reduced by water stress in NT roots, whereas in SAG12-ipt roots it was increased. The 14-3-3E proteins are involved in signal transduction processes such as those that regulate cell elongation (Z Zhang et al., 2010) and are associated with enzymes involved in primary metabolism, many of which were increased in SAG12-ipt roots relative to NT roots under stress, such as nitrate reductase (NR), sucrose synthase, GS, and GADPH (Roberts et al., 2002). Thus, the maintenance of adequate levels of 14-3-3E protein may be a factor contributing to maintenance of signalling capabilities under water stress in SAG12-ipt plants.

Changes in proteins related to stress 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 (Lochmannova 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 H₂O₂ into H₂O and O₂, 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 Fv/Fm, 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 (Lesher 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 lipoygenases (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 P. suzuki 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 Valuce, 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 viability, and overall drought tolerance. Other potentially significant, but less well documented, protein changes occurred in response to CKs or drought in SAG12-ipt plants, such as membrane protein cp-wap13, 14-3-3E, DEAD-box helicase 2, and many proteins with unknown functions. Future evaluation of specific protein changes, particularly those less well documented in regards to CKs or drought stress or those with unknown functions, would be beneficial for more completely revealing the mechanisms of CK regulation of drought tolerance.

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