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RESEARCH PAPER

Photosynthesis, water use, and root viability under water stress as affected by expression of *SAG12-ipt* controlling cytokinin synthesis in *Agrostis stolonifera*

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Abstract

Water stress reduces endogenous cytokinin (CK) content and may inhibit CK production. Maintenance of endogenous CK levels by genetic transformation with *ipt* in leaves and roots undergoing senescence may promote stress tolerance. This study was designed to determine the physiological effects of *ipt* expression on immature and mature leaves and in roots for plants exposed to different levels of water stress for creeping bentgrass (*Agrostis stolonifera*). Plants containing the *ipt* gene, encoding the enzyme adenine isopentenyl phosphotransferase for CK synthesis ligated to a senescence-activated promoter (*SAG12*), and wild-type 'Penncross' (WT) were grown hydroponically in a growth chamber and exposed to water stress by weekly additions of polyethylene glycol 8000 to reduce the growing solution osmotic potential from –0.05 to –0.3, –0.5, –0.7, –1.0, and –1.4 MPa. Immature and mature leaves and roots of *SAG12-ipt* creeping bentgrass were evaluated for *ipt* expression, CK content, leaf relative water content (*RWC*), chlorophyll content (Chl), photochemical efficiency (F_vF_m), osmotic adjustment (*OA*), photosynthesis rate (*Pn*), stomatal conductance (g_s), transpiration (*E*), water use efficiency (*WUE*), carbon isotope discrimination (Δ), and root viability. Expression of *ipt* was detected in all plant parts and a higher CK content, primarily in the form of isopentyladenine (iPa), was found in *SAG12-ipt* plants but not in the WT plants under water stress. Immature leaves exhibited higher iPa and *O*A at all treatment levels. Mature leaves of *SAG12-ipt* plants maintained higher *OA*, *Pn*, Chl, *WUE*, and Δ, whereas g_s and *E* were relatively unaffected compared to the WT. Roots of *SAG12-ipt* plants had higher levels of iPa and greater root viability than the WT. The results demonstrate that expression of *ipt* enhanced the tolerance of creeping bentgrass to water stress, which could be attributed to the positive effects on osmotic adjustment, efficient water use, and maintaining higher photosynthetic rate primarily for mature leaves, as well as increased root viability.

Key words: Cytokinins, drought stress, osmotic stress, *SAG12-ipt*, senescence.

Introduction

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

and by increasing endogenous production of CK through transgenic modification of CK biosynthesis genes or genes regulating CK degradation pathways (Naqvi, 1995).

The CK biosynthesis gene *ipt* encodes for the enzyme isopentyl transferase, which catalyses the rate-limiting first step in *de novo* CK biosynthesis and promotes the formation of isopentenyladenosine-5'-monophosphate (iPa) (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984; McGraw, 1987). The different forms of endogenous *ipt* genes are expressed at relatively low levels in the control condition and during drought stress in several plant species and are highly organ, developmental, or cell type-specific and are down-regulated during stress (Vyroubalova *et al.*, 2009). Generally, it has been demonstrated that *ipt* expression increases endogenous CK or maintains CK levels under stress conditions, thereby delaying leaf senescence and promoting stress resistance in several plant species, such as *Arabidopsis* (*Arabidopsis thaliana*) (Medford *et al.*, 1989; Zhang *et al.*, 2000), lettuce (*Lactuca sativa*) (McCabe *et al.*, 2001), tobacco (*Nicotiana tabacum*) (Rivero *et al.*, 2007), petunia (*Petunia×hybrida*) (Clarke *et al.*, 2004), tall fescue (*Festuca arundinacea*) (Hu *et al.*, 2005), and creeping bentgrass (*Agrostis stolonifera*) (Xu *et al.*, 2009; Merewitz *et al.*, 2010). However, the physiological mechanisms of CK regulation of plant tolerance to water stress remain less well-documented than other water stress regulators such as abscisic acid (Bray, 1993; Kramer and Boyle, 1995; Chaves *et al.*, 2003; Marrion-Poll and Leung, 2006). Analysis of the various organs of different developmental stages of *ipt* transgenic plants with different growth habits and stress resistance mechanisms is critical for further the documentation of the effects of CK on water stress tolerance and the effects of water stress on CK changes.

Previous studies have used senescence activated promoters, such as *SAG12* and *SARK* to auto-regulate or control *ipt* expression to prevent over-production of CK, which may occur with constitutive expression (Medford *et al.*, 1989; Gan and Amasino 1995; Morris, 1995; Rivero *et al.*, 2007; Verdonk, *et al.*, 2008). Transformation of tobacco (*Nicotiana tabacum*) with *ipt*, regulated by a senescence-inducible promoter, resulted in significant improvement in drought tolerance, attributed to the delay in leaf senescence and increases in photosynthesis rates and antioxidant activities (Rivero *et al.*, 2007, 2009). Leaf senescence of mature leaves may be a mechanism for drought survival in order to reduce the surface area for transpiration and to redirect energy reserves to reproductive systems in annual crops where a high yield of the reproductive organs is desirable (Chaves, 2003). However, the maintenance of older leaves by the avoidance of senescence can be beneficial for additional energy produced by maintaining a greater amount of photosynthetic leaves as was found in tobacco (Rivero *et al.*, 2007). The much reduced growth rate of mature leaves largely prevents them from being a sink to draw nutrients and energy from immature leaves or roots that could alternately have gone towards drought survival (Khan, 1981).

For perennial grasses, delaying leaf senescence and maintaining physiological function of both immature and mature leaves under water stress is important for plant biomass production in forage and for the aesthetic appearance in turfgrasses and may improve drought tolerance. Previously, it has been demonstrated that expression of *ipt* in 11 creeping bentgrass transgenic lines maintained higher levels of CK, increased leaf chlorophyll content (Chl), and higher root to shoot ratios after 14 d of drought stress relative to the WT (Merewitz *et al.*, 2010). Cytokinins also regulate many other processes, including stomatal opening, photosynthesis, water relations, and root growth. However, how elevated CK in a perennial grass species such as creeping bentgrass may alter those processes related to water stress tolerance is not clear. Furthermore, limited information is available about the root growth characteristics of *ipt* plants, which is an important factor influencing water uptake under water stress. In the current study, physiological responses of a typical *SAG12-ipt* transgenic line (S41) that exhibited superior drought tolerance compared with the wild-type 'Penncross' of creeping bentgrass were examined in an attempt to elucidate the physiological changes associated with increased CK in the *SAG12-ipt* transgenic plants in both the shoots and roots under water stress. The specific objectives of the study were to determine *ipt* gene expression patterns in leaves and roots during the progression of water stress and to examine the physiological effects of *ipt* expression on immature and mature leaves and in roots for creeping bentgrass exposed to water stress. Physiological assessment focused on leaf senescence (chlorophyll content), water relations (relative water content, osmotic adjustment, stomatal conductance, transpiration, and water use efficiency), and photosynthetic activities (photochemical efficiency and net photosynthetic rate). In addition, since enhanced rooting in the *SAG12-ipt* plants was previously reported, the aim here was therefore to evaluate whether the enhanced rooting was due to delayed root senescence or increased root viability.

Materials and methods

Plant material and growth conditions

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

sulphate (K_2SO_4, 43.568 g^{-1}), magnesium sulphate ($MgSO_4.7H_2O$, 199.65 g l^{-1}), disodium ethylenediaminetetraacetate (Fe(EDTA)Na, 14.684 g l^{-1}), Micronutrients (H_3BO_3, 1.43 g l^{-1}, $MnCl_2.4H_2O$, 0.91 g l^{-1}, $ZnSO_4.H_2O$, 0.11 g l^{-1}, $CuSO_4$, 0.04 g $^{-1}$, $(NH_4)Mo_7\ O_{24}.4H_2O$, 0.01 g l^{-1}). Plants were suspended within 1.27 cm holes in Styrofoam boards that fit within plastic bins (54×42×14 cm in height) that floated on the growth media. The hydroponic solution was aerated with a tube inserted into the solution through the Styrofoam connected to a pump (115 V, 60 Hz, Tetra® Blacksburg, VA). Solution pH was monitored and adjusted as needed every other day and the solution was changed on a weekly basis. Plants were not clipped to allow for adequate stem development for the separation of new and old leaves.

PEG-induced water stress

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

Physiological analysis

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

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

Leaf chlorophyll content (Chl) and photochemical efficiency (F_v/F_m) were measured to evaluate leaf senescence. Total Chl was extracted in the dark for 72 h in dimethyl sulphoxide. The absorbance of the leaf extract was measured at 663 nm and 645 nm with a spectrophotometer (Spectronic Genesys 2; Spectronic Instruments, Rochester, NY, USA). Chl was calculated using the formula described in Arnon (1949). F_v/F_m was evaluated as a ratio of the variable fluorescence (F_v) to the maximal fluorescence (F_m) value determined using a chlorophyll fluorescence meter (Fim 1500; Dynamax, Houston, TX, USA). Leaf clips were used to adapt individual leaves to darkness for 30 min prior to reading the F_v/F_m ratio with the fluorescence meter. Two subsamples were taken per plant at each sampling day.

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

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

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

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

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

Gene expression analysis

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

polymerase enzyme (R001-A, Takara, Madison, WI) set to program rt-pcr50 of the GeneAmp PCR system (9700, Applied Biosystems, Inc., Foster City, CA). PCR control was used to test for contamination.

Experimental design and statistical analysis

The experimental design was a split-plot design with irrigation treatment as the main plots and plant materials as the sub-plots, with four replicates for each irrigation treatment and grass material. Effects of watering treatment, plant materials, and corresponding interactions were determined by analysis of variance according to the general linear model procedure of SAS (Version 9.0; SAS Institute, Cary, NC). Differences between watering treatments and plant means were separated by Fisher's protected least significance difference (LSD) test at the 0.05 probability level.

Results

RT-PCR analysis of ipt *expression*

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

CK content of immature leaves

The iPA content in immature leaves was significantly higher in *SAG12-ipt* plants than in the WT at 0 MPa and during the PEG treatments (Fig. 2). The iPA content declined at –1.4 MPa in all plants, but to a greater extent in WT (65%) than in *SAG12-ipt* plants (24%) (Fig. 2A). DHZR content declined significantly in both WT and transgenic plants during PEG treatment (Fig. 2B). A significantly higher DHZR content in the transgenic plants was detected only at –0.7 MPa, which was 7% higher than in the WT. ZR content was not different between the WT and *SAG12-ipt* plants under the control conditions or throughout the duration of PEG treatment (Fig. 2C). PEG-induced drought stress reduced the ZR content of immature leaves by an average of 71% for both lines. Total CK content (including iPA, ZR, and DHZR) did not differ between the plant lines at the control (0 MPa) level, however, after the osmotic potential of the growth solution was reduced from –0.7 to –1.4 MPa, significant differences were observed. For instance, at –0.7 and –1.4 MPa, total CK in WT plants was reduced by 45% and 64%, respectively, whereas that in the transgenic plants was reduced by 14% and 52%, respectively (Fig. 2D).

CK content of mature leaves

iPA content of mature leaves was greater in the transgenic plants compared to WT at all levels of PEG treatment (Fig. 3A). iPA content declined with PEG-induced water stress, but the rate of iPA loss was slower in the *SAG12-ipt*

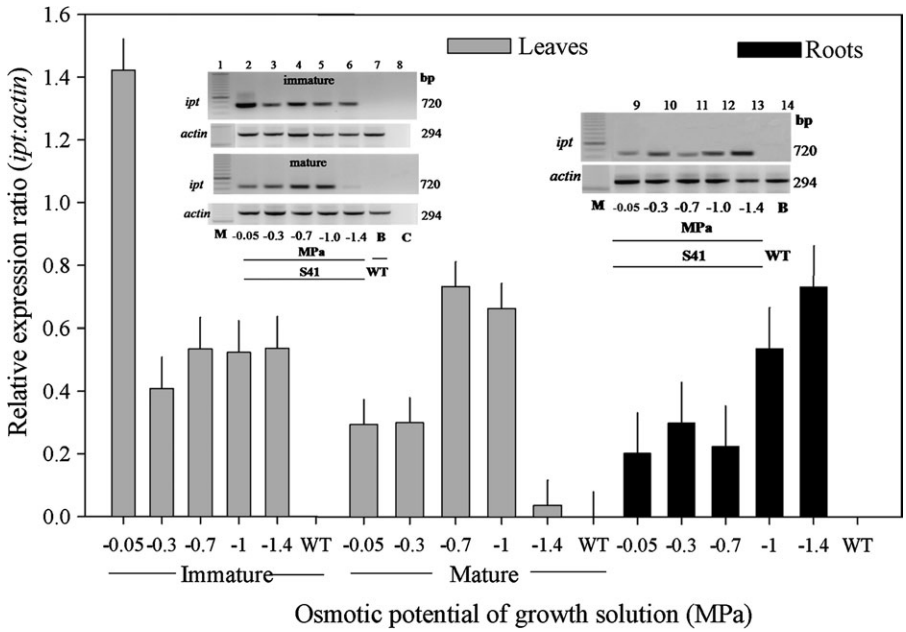

Fig. 1. RT-PCR gel images (inset) and relative expression ratio (bar graph) of *ipt* to the actin internal control in immature leaves, mature leaves, and roots of *SAG12-ipt* creeping bentgrass plants (line S41; lanes 2–8) and wild-type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (–0.05 to –1.4 MPa). C, PCR control, M, molecular weight marker, BP, base pair. Significance at $P \leq 0.05$ is indicated by mean standard error bars ($n=3$).

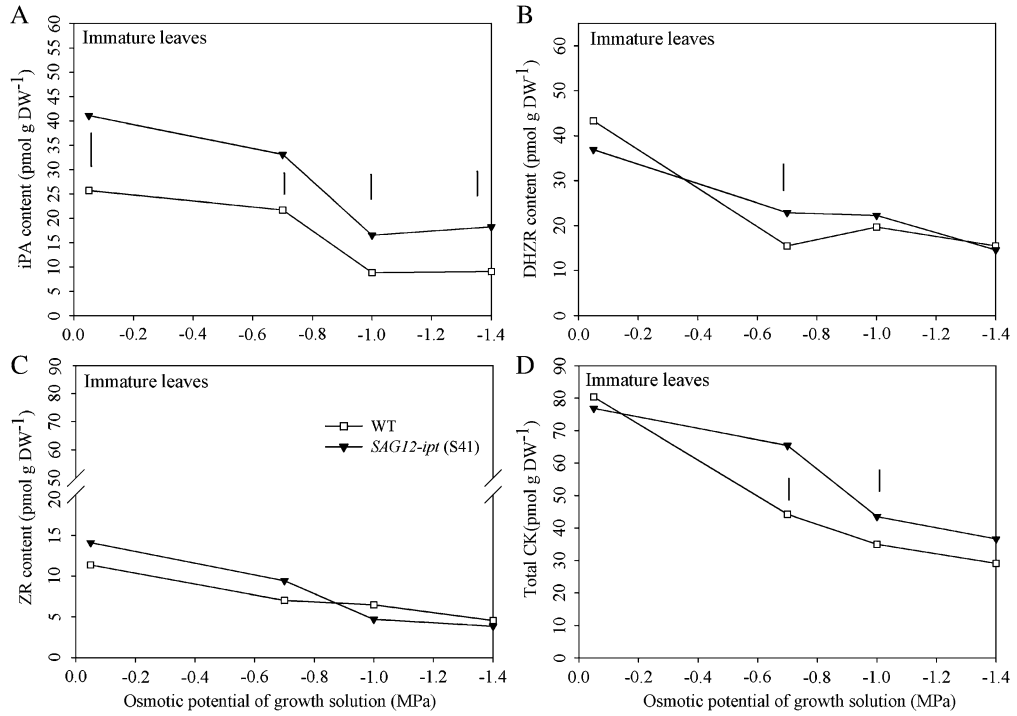

Fig. 2. (A) Isopentyl adenine (iPa) (B) dihydrozeatin riboside (DHZR), (C) zeatin riboside (ZR), and (D) total cytokinin content (CK) (sum of iPa, DHZR, and ZR) of immature leaves (top two leaves not fully expanded) of *SAG12-ipt* creeping bentgrass (line S41) and wild-type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (0 to –1.4 MPa). Vertical bars indicate LSD values where significant differences were detected ($P \leqslant 0.05$) for comparison between plant lines at a given osmotic potential of the growth solution.

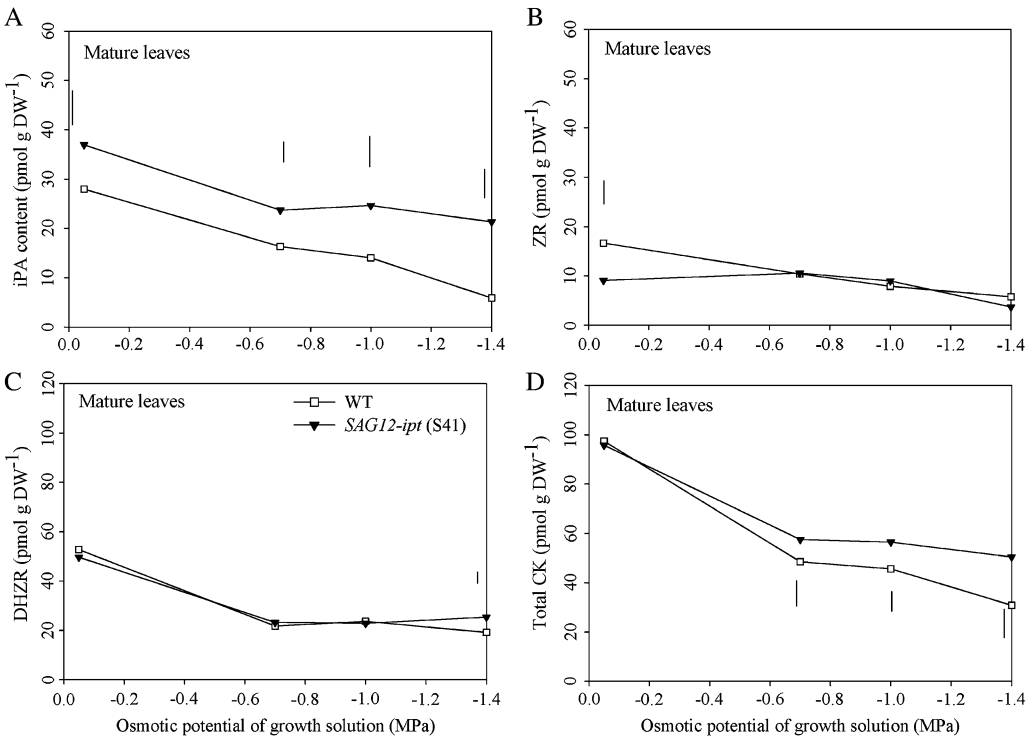

Fig. 3. (A) Isopentyladenine (iPa) (B) dihydrozeatin riboside (DHZR), (C) zeatin riboside (ZR), and (D) total cytokinin content (CK) (sum of iPa, DHZR, and ZR) of mature (fully expanded) leaves of *SAG12-ipt* creeping bentgrass (line S41) and wild-type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (–0.05 to –1.4 MPa). Vertical bars indicate LSD values where significant differences were detected ($P \leqslant 0.05$) for comparison between plant lines at a given osmotic potential of the growth solution.

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

Leaf water status

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

Photosynthetic parameters and carbon isotope discrimination

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

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

Plant lines differed in net photosynthetic rate (*Pn*), stomatal conductance (g_s), transpiration rate (*E*), and water use efficiency (*WUE*, calculated as *Pn*/*E* ratio) of both immature and mature leaves at –0.7 and –1.0 MPa, but not in the control or other PEG treatment levels (Table 1). Values for *Pn* were significantly higher for the transgenic plants compared to the WT at –0.7 and –1.0 in immature leaves, and at –0.7 MPa for mature leaves. Mature leaves of transgenic plants had higher g_s than the WT at the –0.7 MPa treatment, but did not differ from the WT for

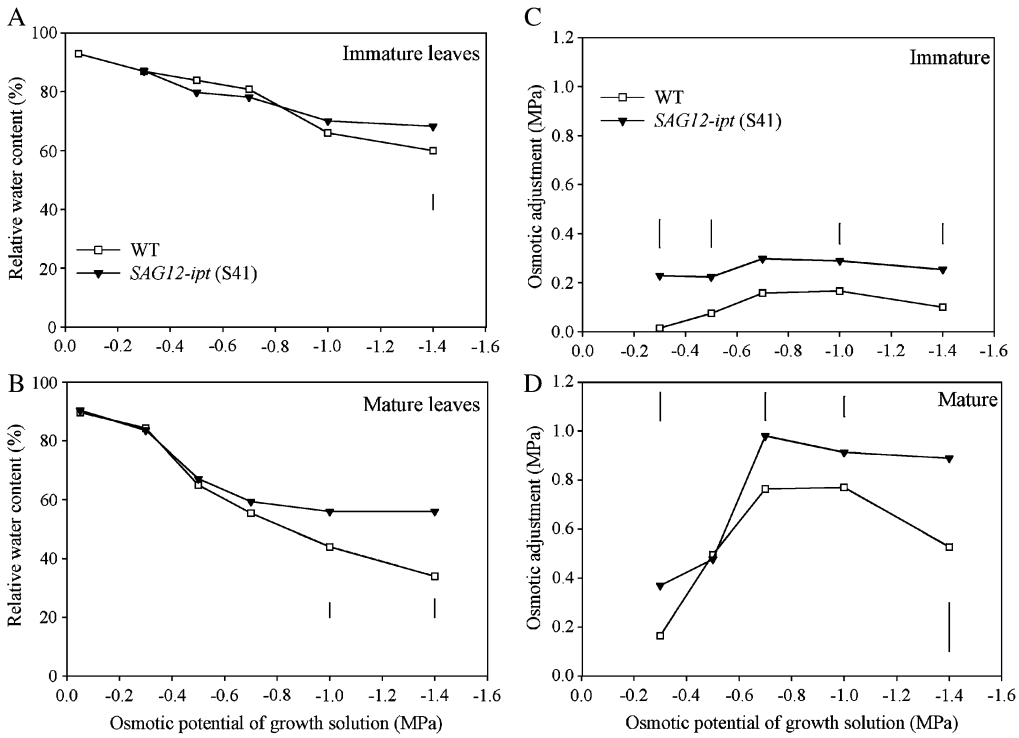

Fig. 4. Relative water content (*RWC*, %) of (A) immature (top two leaves not fully expanded) and (B) mature (fully expanded) and osmotic adjustment (*OA*), calculated as the difference in osmotic potential at between fully rehydrated control and PEG-induced water stress treated leaves, of (C) immature (top two leaves not fully expanded) and (D) mature (fully expanded) leaves of *SAG12-ipt* creeping bentgrass (line S41) and wild-type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (–0.05 to –1.4 MPa). Vertical bars indicate LSD values where significant differences were detected ($P \leq 0.05$) for comparison between plant lines at a given osmotic potential of the growth solution.

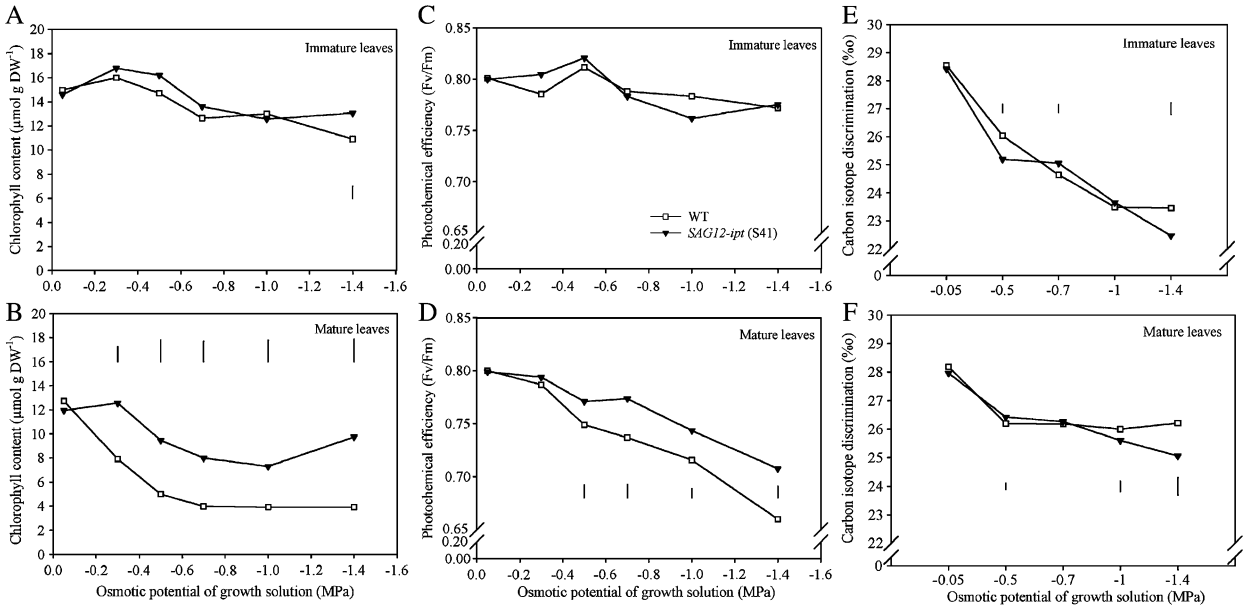

Fig. 5. (A, B) Total chlorophyll content (Chl), (C, D) photochemical efficiency (F_v/F_m), and (E, F) carbon isotope discrimination of immature (top two leaves not fully expanded) and mature leaves of *SAG12-ipt* creeping bentgrass (line S41) and wild-type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (–0.05 to –1.4 MPa). Vertical bars indicate LSD values where significant differences were detected ($P \leqslant 0.05$) for comparison between plant lines at a given osmotic potential of the growth solution.

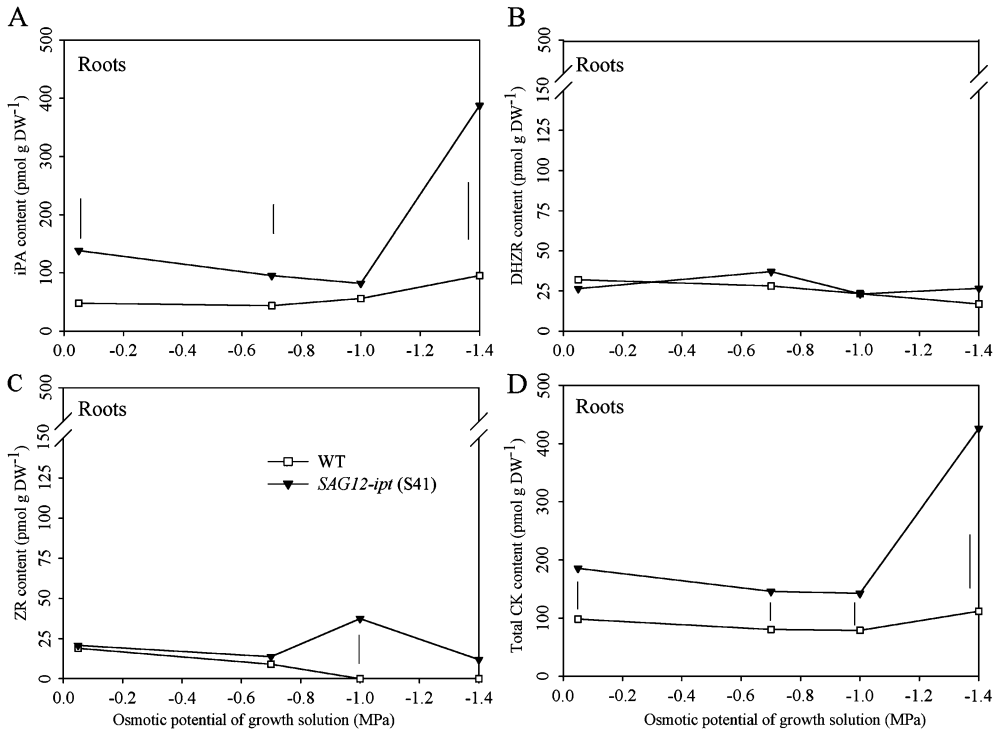

Fig. 6. (A) Isopentyl adenine (iPa) (B) dihydrozeatin riboside (DHZR), (C) zeatin riboside (ZR), and (D) total cytokinin content (CK) (sum of iPa, DHZR, and ZR) of roots of *SAG12-ipt* creeping bentgrass (line S41) and wild-type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (–0.05 to –1.4 MPa). Vertical bars indicate LSD values where significant differences were detected ($P \leqslant 0.05$) for comparison between plant lines at a given osmotic potential of the growth solution.

transpiration rate. The *WUE* of transgenic plants were significantly greater than the WT for immature leaves at –0.7 MPa and for mature leaves at –0.7 and –1.0 MPa. In response to PEG treatment, carbon discrimination ratio (Δ) declined in both WT and transgenic plants, however, transgenic plants had lower Δ values in immature leaves at –1.4 MPa and in mature leaves at –1.0 and –1.4 MPa treatment (Fig. 8).

CK content of roots and root viability

Root iPa content increased in response to PEG treatment to approximately 400% of the control level in transgenic plants and 100% in the WT at –1.4 MPa (Fig. 9). Roots of transgenic plants were significantly higher than the WT roots under control and PEG treatment, and the difference was most pronounced at –1.4 MPa. Root DHZR content was unchanged during PEG treatment and no significant differences in DHZR content were detected between the WT and transgenic plants. ZR content of the transgenic plants was significantly higher at –1.0 and –1.4 MPa, as ZR content of the WT declined to zero in these treatments while it was maintained in the transgenic plants.

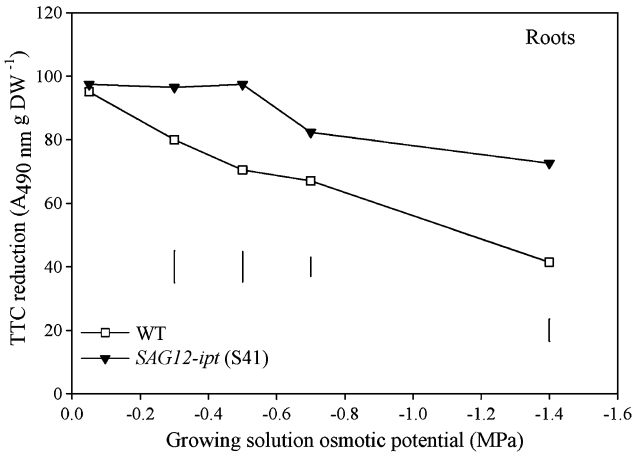

Fig. 7. Root viability, as measured by the triphenyl tetrazolium chloride (TTC) reduction method and the absorbance (A) at 490 nm, of *SAG12-ipt* creeping bentgrass (line S41) and wild-type 'Penncross' (WT) exposed to PEG-induced water stress at various growing solution osmotic potentials (–0.05 to –1.4 MPa). Vertical bars indicate LSD values where significant differences were detected ($P \leq 0.05$) for comparison between plant lines at a given osmotic potential of the growth solution.

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

Discussion

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

The expression of *ipt* was detected in immature and mature leaves as well as in roots under all levels of PEG-induced water stress in transgenic creeping bentgrass. PEG-induced water stress could have evoked an initial senescence response, which activated the *SAG12* promoter for expression of *ipt* in immature and mature leaves and roots of *SAG12-ipt* plants. Drought has been shown to cause

Table 1. Net photosynthesis rate (*Pn*), stomatal conductance (g_s), transpiration rate (*E*), and instantaneous water use efficiency (*WUE*) of immature and mature leaves of wild-type 'Penncross' (WT) and *SAG12-ipt* (S41) plants exposed to PEG-induced drought stress via reduced osmotic potential of the growing solution (–0.7 and –1.0 MPa)

Values followed by the same letter are not significantly different based on Fisher's protected LSD test ($P \leq 0.05$) between plant lines at a given osmotic potential.

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

expression of *ipt* in a previous study (Clarke *et al.*, 2004). Expression of *SAG12-ipt* was also detected under non-stressed conditions in this study and similar findings were reported previously in other plant species, such as in petunia (*Petunia×hybrida*) (Clarke *et al.* 2004), maize (*Zea mays*) (Robson *et al.*, 2004), and tobacco (Rivero *et al.*, 2007), which has been attributed to natural senescence-induced expression. Expression under non-stressed conditions has also been attributed to expression of the endogenous *ipt* genes, since several *ipt* genes have recently been isolated from species such as *Arabidopsis* (Sakamoto *et al.*, 2006), petunia (*sho* gene; Zubko *et al.*, 2002), rice (*Oryza sativa* L., 10 forms of *ipt*; Sakamoto *et al.*, 2006), and maize (*ipt*1,*ipt*2, *ipt*4–8; Brugière *et al.*, 2008). Presumptively, the high level of *ipt* expression observed here under control conditions and in immature leaves of creeping bentgrass could be due to a combination of these factors; however, it seems more likely that it was induced by natural senescence since perennial grass leaves have a relatively high rate of leaf turnover due to the perennial growth habit.

The expression of *ipt* under different levels of PEG-induced water stress was associated with the elevated total CK content in immature and mature leaves and roots of *SAG12-ipt* plants relative to the WT plants. The enhancement of total CK accumulation was primarily due to increased levels of iPa, which is expected since the immediate end-product of the reaction catalysed by *ipt* is iPA (Medford *et al.*, 1989). In immature leaves, the higher *ipt* expression level was generally associated with higher levels of iPa and total CK content. Conversely, mature leaves had relatively low *ipt* expression in the non-stressed condition in conjunction with high CK content but during moderate to severe drought stress, higher expression was accompanied by moderate to low levels of CK. Thus, it is worth noting that *ipt* expression was not always closely correlated to CK content most likely due to drought damage or other factors affecting post-transcription processes and CK abundance. The results suggest that there may be differences in auto-regulation of the *SAG12* promoter and post-trancription processes such as those regulating post-transcription modifications, transcript stability, and translation rates between immature and mature leaves. In addition, CK abundance caused by differences in CK destination, transport activity, and CK degradation due to cellular drought damage may contribute to this CK accumulation difference relative to expression levels between immature and mature leaves. The abundance and activities of cytokinin oxidase (Motyka *et al.*, 1996) and antioxidant enzymes (Synkova *et al.*, 2006) may contribute to these differences, since iPa is the preferred substrate for cytokinin oxidase (Redig *et al.*, 1997) and *ipt* plants have exhibited differential elevation of antioxidant transcripts and enzyme activity in different plant organs of tobacco (Rivero *et al.*, 2007). However, more research is needed to elucidate post-transcriptional phenomenon of *ipt* and how it associates with CK content between immature and mature leaves of creeping bentgrass.

The mechanisms of CK regulation of drought tolerance are not yet fully clear. It is known that CK stimulates stomatal opening, which could help with carbon absorption for photosynthesis, but may cause leaf desiccation under drought stress due to water loss (Chernya'dev, 2005). More recently, it has been found that the timing of increased CK content, the form of CK present, and the balance of hormones may be more critical in determining stomatal responses during drought stress, particularly with *ipt* plants (Pospisilova *et al.*, 2000, 2005). In the current study, immature leaves of the transgenic plants had higher *Pn* under moderate levels of PEG-induced water stress (–0.7 and –1.0) but g_s and *E* did not differ from the WT plants. In addition, mature leaves of the transgenic plants had a lower Δ value relative to the WT throughout drought treatment at –0.5, –0.7, and –1.4 MPa. iPa content, total CK content, and *Pn* were generally higher compared with the WT during the PEG treatment, but there were few differences in g_s or *E* in leaves of transgenic plants; the elevated CK may have affected *Pn* through other mechanisms besides stomatal regulation. Similar results were found in water-stressed *ipt* tobacco since stomatal conductance was largely the same between *ipt* and WT plants (Rivero *et al.*, 2009). They concluded that maintenance of photosynthesis rates in *ipt* tobacco was possibly due to non-stomatal traits such as increased photorespiration, which promotes photosynthesis under drought stress by providing RUBP and other beneficial metabolites (Wingler *et al.*, 2000). This was evident to Rivero *et al.* (2009) by the increased level of transcripts coding for enzymes in the photorespiration pathway, increased metabolites generated by photorespiration, greater antioxidant content, and an increase in the CO_2 compensation point in *ipt* plants compared with the WT. These mechanisms may also be a factor in *SAG12-ipt* creeping bentgrass observed indirectly in this study as higher *Pn* in the mature leaves of the transgenic plants, increases in chlorophyll content, and greater F_v/F_m. In addition, differences in *Pn* contributed to differences in *WUE* between plants lines. This is in conjunction with the differences in Δ values between the WT and transgenic plants, as transgenic plants had lower Δ than the WT during most of the PEG treatments. In Kentucky bluegrass (*Poa pratensis*), higher values of *WUE* have been associated with less wilting, greater TQ, and superior water relations under drought stress (Ebdon and Kopp, 2004). In several cool season grasses, low Δ was associated with higher instantaneous water use efficiency; plants with low Δ had higher water potential, solute potential, and turgor pressure, exhibiting a better capacity for growth under drought stress (Johnson and Basset, 1991). Thus, most likely the *ipt* gene and increased CK may enhance metabolic activities that may promote photosynthetic activity without increasing stomatal conductance or transpiration rate, thereby increasing water use efficiency, especially in immature leaves. CK involvement in regulating *Pn*, g_s, *E*, *WUE*, and Δ under drought stress deserves additional investigation since increases in CK is not always associated with increases in g_s as previously proposed and some studies have shown a decrease in g_s in response to elevated CK (Pospisilova *et al.*, 1997, 1998).

Our results show that CK may have some involvement in regulating water relations and OA, as *SAG12-ipt* plants had better *OA* and retained more water, particularly in mature leaves. However, it is not clear whether this is a direct, primary result of altered *OA* and water status or a secondary effect of improved water status due to increased overall physiological activities or photosynthetic efficiency. Recent literature in the role of CK in regulating osmotic adjustment is conflicting. In transgenic potato that accumulate CKs (*Solanum tuberosum*), *RWC* and *OA* seemed to be unaffected by CK levels (Siffel *et al.*, 1992; Popsisilova *et al.*, 2000). Conversely, *ipt* plants have also been shown to have enhanced levels of osmolytes such as proline, which would typically have an effect on OA. The changes in proline in this study were not always associated with reduced wilting of *ipt* plants (Thomas *et al.*, 1995). Thus, due to the complexities of comparing different plant species under different promoters expressing *ipt*, a clear role of CK in regulating water relations is still not fully elucidated. It has been suggested that CK may cause a slight osmotic response similar to a small degree of salt stress to allow increased tolerance via adaption for subsequent stress such as drought (Thomas *et al.*, 1995; Pospisilova *et al.*, 2000). Our results seem to be consistent with this explanation since CK levels were higher and *OA* was increased even under a low level of water stress (–0.3 MPa), and, consistently, the transgenic plants, compared to WT, in both immature and mature leaves, even when differences in other parameters such as *RWC* or F_v/F_m were not yet observed.

Effects of *ipt* expression on the physiological responses to PEG-induced water stress varied between mature and immature leaves. Generally, the positive effects of *ipt* expression on the physiological parameters examined here were more pronounced for mature leaves, whereas fewer differences occurred for immature leaves between the transgenic and WT plants. Significant differences between *SAG12-ipt* and WT in *Pn*, *WUE*, and Δ in immature leaves were observed earlier during moderate water stress (–0.7 and –1.0 MPa), whereas differences between *SAG12-ipt* and WT in Chl, F_vF_m, *E*, *RWC*, and *OA* in immature leaves, were not observed until more severe water stress levels were reached (–1.0 to –1.4 MPa). In mature leaves, differences between *SAG12-ipt* and WT in most of these parameters started at approximately –0.5 MPa. Senescence was delayed in mature leaves of *SAG12-ipt* plants compared with mature leaves of WT, indicated by maintenance of higher Chl and F_vF_m during water stress. Delay in leaf senescence is significant for plants to adapt to water stress, as the first symptoms of senescence are seen as a loss of chlorophyll and chloroplasts as the plant degrades leaf mesophyll cells for nutrient remobilization (Lim *et al.*, 2007). The results of this study show that the expression of the *SAG12-ipt* in creeping bentgrass at highly detectable levels was sufficient to maintain levels of CK in immature and mature leaves and increase root CK of creeping bentgrass subjected to water stress. It may be assumed that enough CK was produced to overcome degradation of the free forms of CK by cytokinin oxidases, which are up-regulated in the drought response in most plant organs (Vyroubalova *et al.*, 2009).

Root *ipt* expression analysis revealed a gradual increase in transcript abundance with the severity of PEG-induced water stress, which corresponded to an increase in CK levels at –1.4 MPa osmotic potential. Despite the increase in *ipt* transcription during moderate (–0.5 to –1.0 MPa) water stress, a corresponding increase in root CK content during moderate stress was not observed, which could be due to sustained CK transport to the leaves. However, in roots exposed to –1.4 MPa PEG treatment, a sharp increase in CK was observed, which could be due to the interruption of CK transport from roots to other parts of a plant under severe water stress. A decline of root CK content during moderate water stress followed by an accumulation of CK in the roots during severe water stress was also found in *SAG12-ipt* tobacco (Havlova *et al.*, 2008). The results of the current study suggest that this could be due to reduced CK transport activity as opposed to a decline in root viability, as *SAG12-ipt* root viability was significantly higher at –1.4 MPa relative to the WT plants. Other work has also suggested that CK accumulates in roots under severe drought stress due to reduced xylem transport of CK or possibly due to a decrease in CKX activity (Novakova *et al.*, 2007). Kudoyarova *et al.* (2006) reported that loading of CK into the xylem was decreased in tomato plants exposed to dry soils. Similar results were found in response to heat stress, *SAG12-ipt* bentgrass exhibited increased iPa in the roots by approximately ten times the amount in the roots at optimal temperature of 20 °C, whereas the leaves had a less dramatic increase in iPa content in response to temperature, however, leaf iPa content was maintained at higher levels in the transgenic lines compared with the WT. Similar to our results, a less dramatic change was observed for the other forms of CK such as ZR and DHZR compared with iPa in response to temperature and the transgene in this study (Xu *et al.*, 2009).

Limited information is available about the role of CK in root senescence and mortality under drought-stressed conditions. Our results indicate that increased endogenous CK in the *SAG12-ipt* plants was associated with higher root viability. The results show that CK may be involved in regulating root mortality, since *SAG12-ipt* plants had increased root viability under water-stress conditions. Promoting root growth and viability were previously reported in a study with exogenous applications of different forms of CKs on creeping bentgrass exposed to heat stress (Zhang and Ervin, 2008). The mechanisms for root survival of water stress associated with *ipt* expression are not known. It is possible that the delay in leaf senescence and the maintenance of higher photosynthesis of the *SAG12-ipt* plants under water stress could have maintained adequate carbon available to transport to the roots to maintain root growth or survival. Alternatively, CK may directly be involved in the regulation of root mortality; however, this is not yet clear. Previous research has indicated CK may play a direct role in root stress signalling by stimulation of root antioxidants to reduce root lipid peroxidation, which

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

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

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