Effects of SAG12-ipt and HSP18.2-ipt Expression on Cytokinin Production, Root Growth, and Leaf Senescence in Creeping Bentgrass Exposed to Drought Stress

Emily B. Merewitz, Thomas Gianfagna, and Bingru Huang
Department of Plant Biology and Pathology, Rutgers University, New Brunswick, NJ 08901

ABSTRACT. Drought stress is a widespread abiotic stress that causes a decline in plant growth. Drought injury symptoms have been associated with an inhibition in cytokinin (CK) synthesis. The objectives of this study were to investigate whether expression of a gene (ipt) encoding the enzyme adenine isopentenyl phosphotransferase for CK synthesis ligated to a senescence-activated promoter (SAG12) or a heat shock promoter (HSP18.2) would improve drought tolerance in creeping bentgrass (Agrostis stolonifera) and to examine shoot and root growth responses to drought stress associated with changes in endogenous production of CK, and the proportional change in CK and abscisic acid (ABA) due to ipt transformation. Most SAG12-ipt and HSP18.2-ipt transgenic lines exhibited significantly higher turf quality, photochemical efficiency, chlorophyll content, leaf relative water content, and root:shoot ratio under drought stress than the null transformant or the wild-type ‘Penncross’ plants. Transgenic lines that had better growth and turf performance generally had higher CK content and a higher CK-to-ABA ratio, although the direct correlation of CK and ABA content with individual physiological parameters in individual lines was not clear. Our results demonstrated that expressing ipt resulted in the improvement of turf performance under drought stress in creeping bentgrass in some of the transgenic plants with SAG12-ipt or HSP18.2-ipt, which could be associated with the suppression of leaf senescence and promoting root growth relative to shoot growth due to the maintenance of higher CK level and a higher ratio of CK to ABA.

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

To study the effects of CK metabolism on stress tolerance and the mechanisms of CK regulation of stress tolerance, two approaches have been employed: exogenous application of CK and transgenic modification of endogenous CK levels. Generally, plants maintaining or exposed to higher levels of CK, either by alterations of endogenous production by transgenic methods or by exogenous application, exhibit improved tolerance to different stresses. For example, creeping bentgrass plants that were treated with a CK injection into the root zones showed increases in TQ and photochemical efficiency (Fv/Fm) largely due to the alleviation of heat-induced root mortality and increased antioxidant activity (Liu et al., 2002; Liu and Huang, 2002). Likewise, Zhang and Ervin (2004) demonstrated that creeping bentgrass showed improved TQ under drought stress when treated with an exogenous application of a seaweed extract containing CK. However, the exogenous application of hormones does not always provide the same physiological effects as changes in endogenous levels of hormones (Okamoto et al., 2010). Thus, internal modifications of CK levels may be more useful for understanding how CK regulates drought tolerance. The CK gene used in this study encodes adenine isopentenyl transferase (ipt), which catalyzes the formation of isopentenyadenosine-5’-monophosphate from 5’AMP and isopentenylypyrophosphate, a key enzyme involved in the rate-limiting step leading to de novo CK biosynthesis (Medford et al., 1989; Morris, 1995). Transgenic plants expressing the ipt gene exhibit increased tolerance to different stresses in some plant species, including drought in petunia (Petunia hybrida) (Dervinis, 1999), lettuce (Lactuca sativa) (McCabe et al., 2001), and tobacco (Nicotiana tabacum) (Rivero et al., 2007), flooding in arabidopsis (Arabidopsis thaliana) (Huynh et al., 2005; Zhang et al., 2000), cold in tall fescue (Festuca...
arundinacea) (Hu et al., 2005), and nutrient deficiency in tobacco (Jordi et al., 2000). In ipt transgenic lettuce, the observed increases in drought tolerance of the transgenic plants were attributed to hexose accumulation (McCabe et al., 2001). Rivero et al. (2007, 2009) reported that ipt transgenic tobacco exhibited improved drought tolerance due to delayed leaf senescence, changes in photosynthesis, protection of photosynthesis, and increased water use efficiency. Havlova et al. (2008) transformed tobacco with a gene encoding transzeatin O-glucosyltransferase (ZOG1) to increase endogenous CK O-glucosides, a storage form of CK, and found delayed leaf senescence of older leaves, decreases in cytokinin oxidase activity during drought stress, and improvement in postdrought recovery compared with wild-type controls. The benefits of elevated CK levels under drought stress in a perennial grass species maintained under turf conditions where leaf senescence is a primary concern for TQ have not yet been evaluated and may be different from annual crops such as tobacco and lettuce. In addition, the senescence of older leaves is known to be a drought survival mechanism similar to dormancy in many crop species. This mechanism may be desirable in some plants as a way to redirect energy reserves to younger leaves or toward plant reproduction, thus increasing yield or for survival at the whole plant level. However, it has also been shown that maintenance of older leaves by avoidance of senescence is beneficial for additional energy produced by a greater amount of photosynthetic source leaves (Rivero et al., 2007). Furthermore, due to the cessation of significant growth relative to younger leaves, older leaves do not typically act as much of a sink to draw nutrients away from a plant, reducing energy that could have gone toward drought tolerance mechanisms (Khan, 1981). In addition, perennial turfgrass species performance is not based on yield but on aesthetic appearance for which leaf senescence is undesirable. Limited information is available about the root growth characteristics of ipt plants, which is an important factor influencing water uptake under drought stress. With an aim to examine the effects of CK effects on drought performance in perennial turfgrass species, we transformed a widely used cool-season turfgrass species, creeping bentgrass, using the ipt gene ligated to a senescence-associated promoter, SAG12 (Gan and Amasino, 1995) and a heat shock promoter, HSP18.2 (Takahashi and Komeda, 1989). The senescence- and stress-inducible promoters circumvent the abnormal growth problems associated with the overproduction of CK in transgenic plants containing the ipt gene driven by constitutive promoters (Dansanko et al., 2003; Gan and Amasino, 1995; Schnablava et al., 2006; Yoshida and Shinmyo, 2000). In previous studies, SAG12-ipt transgenic creeping bentgrass exhibited improved growth under heat stress (Xu et al., 2009) and nutrient deficiency (Zhang et al., 2010) in association with increased tiller production, root growth, and root:shoot ratio. The objectives of this study were to investigate whether expression of the ipt gene-promoting CK synthesis driven by senescence- and/or stress-inducible promoters would improve drought performance in creeping bentgrass, and to examine shoot and root growth responses to drought stress associated with changes in endogenous production of CK and the ratio of CK and ABA due to the ipt transformation.

**Materials and Methods**

**PLANT MATERIAL AND GROWTH CONDITIONS.** Transgenic plants were developed by the agrobacterium (Agrobacterium tumefaciens) transformation method as described in Xing et al. 2010 and Xu et al. 2009. Plant materials included SAG12-ipt transgenic lines (S7, S8, S16, S25, S32, S37, S40, S41, S55, S97, and S99), HSP18.2-ipt transgenic lines (H13, H27, H29, H31, H37, H42, and H43), the wild-type cultivar ‘Penncross’ (WT), and a null transformant (NT) control line of ‘Penncross’ that was transformed with an empty plasmid vector without the ipt gene. The transgenic plant lines used in this study were verified, by northern analysis, to be transformed and to contain the ipt gene, whereas the WT and NT plant lines did not contain the transgene, as shown in Xu et al. 2009. In addition, all material has been clonally propagated since northern confirmation analysis to negate any possibility of transgene loss due to sexual reproduction or recombination. Plant materials were established in eight large plastic containers (54 cm long, 42 cm wide, and 14 cm tall) filled with fine sand (0.125 mm particle size) with ≈10 individual plants from the control and each transgenic line. Plants were grown in a controlled environment growth chamber (GC15; Environmental Growth Chambers, Chagrin Falls, OH) and were allowed to establish for 4 weeks before watering treatment imposition. The growth chambers were set to regulate chamber conditions at a 12-h photoperiod, 50% relative humidity, 500 μmol·m⁻²·s⁻¹ photosynthetic photon flux (PPF), and a day/night temperature of 23/20 °C. Plants were watered well and were fertilized with a controlled-release fertilizer (19N–2.6P–10K; Scotts, Marysville, OH) once during plant establishment in the greenhouse and once before water treatment in the growth chamber. Plants were maintained at ≈3 cm height by hand clipping weekly during the establishment period, but were not trimmed during drought stress treatment.

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

**MEASUREMENTS.** Soil volumetric water content was determined with the time domain reflectometry method (Topp, 1980) (Trase; Soil Moisture Equipment, Santa Barbara, CA). Two-pronged waveguide probes 20 cm in length were buried horizontally in the middle of the root-zone media in each container and measurements were taken periodically during the 14-d treatment period. Overall turf performance was evaluated by visually rating TQ. Turf quality was visually rated every 2 d based on turf uniformity, color, and density on a scale of 1 to 9 with 1 being brown and desiccated turf, 6 being the minimal acceptable level, and 9 being green and dense turf (Turgeon, 2008). Total root length and biomass and total shoot biomass were determined at the end of drought stress (14 d) by destructive sampling. Roots were washed free of sand and separated from shoots at the crown. Total root length was calculated by separating the fresh roots on a flatbed scanner (4490; Epson, Long Beach, CA) and the total length was calculated with WinRhizo software (Regent Instruments, Loretteville, Canada). Subsequently, all plant biomass was dried in an 80 °C oven for 72 h for dry weight (DW) determination. Root-to-shoot ratio was calculated as the ratio of root DW to shoot DW that included all tissues of the whole plant.
Relative water content (RWC) of leaves was measured as an indicator of leaf hydration status. Leaf RWC was calculated based on fresh (FW), turgid (TW), and DW of ≈0.1 g of leaf samples using the following formula: (FW – DW)/(TW – DW) × 100. Leaf FW was determined on a mass balance immediately after being excised from the plants. Turgid weights were determined after soaking the leaves in deionized water for 12 h in a closed petri dish at 4 °C and weighing them immediately after being blotted dry. Leaves were then dried in an 80 °C oven for at least 72 h before being weighed for DW (Barrs and Weatherley, 1962).

Leaf Chl content and Fv/Fm were measured to evaluate leaf senescence. A hand-held leaf Chl meter (SPAD-502; Spectrum Technologies, Plainfield, IL) was used to measure Chl content on two subsamples taken per plant. The Chl meter gives an index of total leaf Chl content. The index values were converted to Chl content and were expressed as milligrams per gram DW using a standard curve constructed with actual Chl content against the index values. Chlorophyll of leaves for the standard curve was extracted in dimethyl sulfoxide, and the absorbance was measured at 663 and 645 nm with a spectrophotometer (Genesys 2; Spectronic Instruments, Rochester, NY). The content of Chl was calculated using the formula described in Arnon (1949). Photochemical efficiency was evaluated as a ratio of the variable fluorescence (Fv) to the maximal fluorescence (Fm) value determined using a Chl fluorescence meter (Fim 1500; Dynamax, Houston). Leaf clips were used to adapt individual leaves to darkness for 30 min before reading the Fv/Fm ratio with the fluorescence meter. Two subsamples were taken per plant at each sampling day.

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

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

Results

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

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

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

**TOTAL CHL CONTENT.** Leaf Chl content did not vary between plant lines and remained constant under well-watered conditions (Fig. 4, A and B). Among plant lines exposed to drought stress, significant differences occurred after 2, 6, and 12 d of drought (Fig. 4, A and B). Leaf Chl content declined in all plant lines in response to drought stress, but the declines in SAG12-ipt lines were less pronounced than for NT and WT plants. The Chl content of NT and WT plants declined by an average of 68% at 12 d of drought, whereas the Chl content of SAG-ipt lines declined by an average of 50%. Transgenic lines H27, S39, S25, and S41 had the greatest amount of Chl, and NT plants had the lowest Chl content under drought stress.

**Fv/Fm.** Under well-watered conditions, no significant differences in Fv/Fm were detected between the plant lines, which maintained an average of 0.80 throughout the duration of the experimental period (Fig. 5, A and B). Drought stress caused a significant decline in Fv/Fm in all plant lines (Fig. 5B). HSP18.2-ipt and SAG12-ipt lines exhibited variation in Fv/Fm, and several ipt lines maintained significantly higher Fv/Fm levels compared with NT and WT plants at 6, 9, and 14 d of

![Fig. 3. Turf quality (TQ) of the null transformant (NT), wild-type 'Penncross' (WT), HSP18.2-ipt (H lines), and SAG12-ipt (S lines) of creeping bentgrass exposed to well-watered conditions (A and C) and drought stress (B and D). TQ was determined using a visual rating system with 1 = brown and desiccated turf, 6 = minimal acceptable quality, and 9 = green and dense turf (Turgeon, 2008). Vertical bars indicate LSD values (P ≤ 0.05) for comparison between plants lines at a given day of treatment where significant differences were detected.](image-url)
drought. By 14 d of drought stress, all transgenic lines had significantly higher Fv/Fm than the NT line.

**ROOT GROWTH AND ROOT:SHOOT RATIO.** Plant lines H13, H29, H31, S16, S25, S32, S43, S55, S7, S97, and S99 had significantly higher total root biomass than the WT under drought stress. The same lines, with the addition of line S37 and the exception of lines H29, H31, S7, and S97, exhibited greater total root length, which can most likely be attributed to the additional root biomass. The root:shoot ratio was analyzed to normalize differences between transgenic-ipt and control lines under optimal conditions such as due to differential tiller numbers (Xu et al., 2009). Root:shoot ratios were generally higher in transgenic-ipt lines compared with NT and WT after 14 d of drought (Fig. 6C); for example, lines S25 and S7 had root:shoot ratios of ≈0.25, whereas the average root:shoot ratio of NT and WT was ≈0.075. The root:shoot ratio averaged ≈0.48 for the SAG12-ipt, NT, and WT under well-watered conditions (Fig. 6). At 14 d of drought stress, significant differences in root:shoot ratio were observed between plant lines (Fig. 6). Transgenic-ipt lines H29, H31, S16, S25, S32, S43, S7, S97, and S99 had significantly higher root:shoot ratios compared with the NT and WT plants. The highest root:shoot ratio in drought-stressed plants was found in transgenic line S25 and S7, at ≈0.25, whereas the lowest ratio was in NT plants, at ≈0.05.

**LEAF iPA AND ABA CONTENT.** Leaf iPA content of well-watered plants did not differ significantly between plant lines, which averaged ≈30 pmol·g⁻¹ DW in leaves (Fig. 7A, threshold). After 14 d of drought, iPA content declined in all plants. Seven of the 12 SAG12-ipt lines (S25, S37, S40, S41, S43, S7, and S99) had a significantly higher leaf iPA content compared with NT and WT, although variation in iPA accumulation existed among these transgenic-ipt lines (Fig. 7A). The SAG12-ipt lines that were significantly different from NT and WT had an average iPA content more than four times higher, at 9.0 pmol·g⁻¹ DW in transgenic-ipt lines compared with 2.2 pmol·g⁻¹ DW in WT and NT plants. Slight increases in iPA content were found between the HSP18.2-ipt line and the control lines; however, these were not statistically different. Under well-watered conditions, leaf ABA content was ≈25 pmol·g⁻¹ DW (Fig. 7B). Drought stress resulted in an accumulation in leaf ABA content above this control level. Transgenic-ipt lines H13, H29, H42, S25, S32, S40, S41, S55, S7, S97, and S99 maintained leaf ABA levels significantly lower than NT and WT plants at 14 d of drought stress (Fig. 7B).

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

**Discussion**

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

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

The increases in total root biomass production in our study may be due to increases in root production associated with the stimulation of tiller formation in *SAG12-ipt* transgenic creeping bentgrass, as reported by Xu et al. (2009). Moreover, Aloni et al. (2006) showed that CK played a significant role in promoting root development, differentiation, and architecture. Specifically, they found that elevated root CK levels in root tips, as controlled by *ipt* genes, may cause root apical dominance and may allow primary roots to reach water in deeper soil layers. Increased apical dominance promoted primary root growth as opposed to lateral roots. The maintenance of greater root:shoot ratios under drought stress could be at least in part due to enhanced root survival, root production, and/or root elongation due to the expression of *ipt* in creeping bentgrass under drought stress. Root:shoot ratio has been shown to be an effective selection method in breeding for drought tolerance of perennial turfgrasses such as tall fescue (Karcher et al., 2008). In addition, our results are in agreement with other studies in creeping bentgrass in that an exogenous application of CK (Liu and Huang, 2002) and the presence of *SAG12-ipt* (Xu et al., 2009) promoted root growth during heat stress conditions.
Endogenous leaf iPA content was lower under heat stress than the values for well-watered plants in creeping bentgrass (Xu et al., 2009), but SAG12-ipt plants still had higher iPA content than NT. Similarly, in this study, total additive iPA content, including that found in leaves and roots, was maintained at higher levels in ipt plants relative to the NT and WT controls under drought stress. Plants that had significantly higher levels of leaf iPA generally had better TQ ratings, greater Chl content, and higher RWC and Fv/Fm by 14 d of drought than nontransgenic lines and thus had lower levels of drought-induced leaf senescence, although not all transgenic lines that had higher iPA content exhibited improved drought tolerance, as discussed above, or there seemed lack of a direct correlation between iPA content and turf growth when comparing individual transgenic lines. It is possible that other forms of CK such as zeatin riboside and dehydrozeatin riboside may be changed as the result of the transformation, which may account for the variations between transgenic lines. However, the hormone balance of the plant lines may better explain the improvements in physiological attributes under drought stress. This and differences in iPA translocation may be particularly true for the Hsp18.2-ipt lines that had higher root iPA content, but did not accumulate iPA to levels higher than the nontransgenic lines in the leaves. However, considering that iPA is a predominant form of CK in perennial grass species as previously reported (Xu et al., 2009), this study only quantified iPA. Nevertheless, our findings are consistent with previous work done to evaluate exogenous applications of CK, where increased levels of leaf iPA were associated with greater drought tolerance (Zhang and Ervin, 2004). Differences in drought tolerance of bentgrass species have been associated with differences in total CK content in the plant (DaCosta and Huang, 2007). Comparing iPA content in leaves and roots, it seems that at least several transgenic lines such as S32 and S55, S8, and S92 that did not exhibit higher iPA than the NT and WT in leaves had significant increases in roots. Additionally, the poorly performing line H43 most likely had an inadequate hormone balance because it had a relatively high root ABA content, a low leaf ABA content relative to NT, a high root iPA content, and relatively low leaf iPA content relative to the other lines. The higher amount of ABA in the shoots of H43 relative to the roots may indicate leaf cell damage despite ABA signaling because efficient ABA translocation is required for ABA signaling and an adequate drought tolerance response (Liu and Huang, 2005), and an accumulation of ABA has been shown to occur in less drought-tolerant plants, as discussed below (DaCosta and Huang, 2007). The high total plant iPA of H43 may indicate that the transgene was being expressed at too high of a level. However, further expression analysis studies would be needed to confirm such a conclusion. It is well known that CK are commonly found in the xylem and are thereby transported from the roots, where they are primarily synthesized, to the shoot (Letham and Palni, 1983). Our results suggest that the translocation pattern of iPA between roots and leaves may have been altered in some transgenic plants, which may have caused higher root iPA and may have affected the ABA:CK ratio, resulting in the increases in root growth. However, this cannot be directly concluded because translocation and differences in CK conversion among all forms was not explicitly measured. Alternatively, other mechanisms could be possible because CK have been shown to be involved in other root processes such as promoting vascular differentiation (Aloni et al., 2006), which could have allowed for healthier roots under drought stress and therefore the greater ability of plants to maintain root growth under stress. Alternative to our results, one could argue that CK in the form of iPA is known to cause stomatal opening and reduced root growth, which would reduce drought resistance characteristics. However, it has been found that the timing of increased CK content, the form of CK present, and the balance of hormones may be more critical in determining stomatal responses during drought stress (Pospisilova et al., 2000, 2005).

Drought stress can lead to an increase in ABA accumulation in various plant species, including creeping bentgrass (DaCosta and Huang, 2007). Most of the transgenic lines had lower ABA content in leaves and roots than the non-transgenic plants. ABA has been associated with the promotion of drought responses, such as stomatal closure, that lead to photosynthesis inhibition (Blackman and Davies, 1983). Lower levels of ABA accumulation have been correlated to drought tolerance in different perennial grass species due to less cellular damage, most likely achieved by alternative drought-adaptive mechanisms (DaCosta and Huang, 2007; Volaire et al., 1998; Wang et al., 2003). Reduced accumulation of ABA may reflect less drought injury in roots and shoots associated with the increases in CK production in transgenic plants. In contrast, some research has reported increased ABA content being associated with greater drought tolerance (Rivero et al., 2007, 2009). Thus, multiple dynamic mechanisms are involved and are not yet fully clear. The higher ABA content in leaves of NT and WT may induce stomatal closure and result in limited photosynthesis during drought stress. The ratio of iPA to ABA was generally higher in leaves and roots of transgenic plants than in the WT and NT plants. Hormone interactions are dynamic because concentrations of other hormones and their proportion between roots and shoots may influence plant growth and development, including leaf senescence (Naqvi, 1995) and stomatal aperture (FuBeder et al., 1992). In a study with Medicago sativa, plants with a lower ABA content in roots and a higher CK-to-ABA ratio in leaves, as well as higher leaf CK concentrations, maintained photosynthetic activity, leaf conductance, and transpiration flux under drought stress (Goicoechea et al., 2006). The improved drought performance along with the increase in CK-to-ABA ratio in SAG12 and HSP-ipt plants suggests that CK may have an important role in the regulation of drought tolerance in creeping bentgrass through changing the accumulation and the balance with ABA.

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

**Literature Cited**

Aloni, R., E. Aloni, M. Langhans, and C.I. Ullrich. 2006. Role of cytokinin and auxin in shaping root architecture: Regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Ann. Bot. (Lond.) 97:883–893.
Aron, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24:1–13.

Bars, H.D. and P.E. Weatherly. 1962. A re-examination of the relative turgidity techniques for estimating water deficits in leaves. Aust. J. Biol. Sci. 15:413–428.

Bettany, A.J.E., S.J. Dalton, E. Timms, and P. Morris. 1998. Stability of transgene expression during vegetative propagation of protoplast derived tall fescue (*Festuca arundinacea* Schreb.) plants. J. Exp. Bot. 49:1797–1804.

Blackman, P.G. and W.J. Davies. 1983. The effects of cytokinins and ABA on stomatal behavior of maize and *Commelina*. J. Exp. Bot. 34:1619–1626.

Bray, E.A. 1993. Molecular responses to water deficit. Plant Physiol. 103:1035–1040.

Chaves, M.M., J.P. Maroco, and J.S. Pereira. 2003. Understanding plant responses to drought: From genes to whole plant. Funct. Plant Biol. 30:239–264.

Clark, D.G., C. Dervinis, and J.E. Barrett. 2004. Drought-induced leaf senescence and horticultural performance of transgenic *PSAG12-IPT* petunias. J. Amer. Soc. Hort. Sci. 129:93–99.

DaCosta, M. and B. Huang. 2007. Drought survival and recuperative ability of bentgrass species associated with changes in abscisic acid and cytokinin production. J. Amer. Soc. Hort. Sci. 132:60–66.

Dansanko, T., K. Kato, M. Sekine, K. Yoshida, and A. Shimmyo. 2003. 5′-Untranslated region of the HSP18.2 gene contributes to efficient translation in plant cells. J. Biosci. Bioeng. 95:52–58.

Davies, W.J., F. Tardieu, and C. Trejo. 1994. How do chemical signals work in plants that grow in drying soil? Plant Physiol. 104:309–314.

Dervinis, C. 1999. Genetic transformation of *Petunia hybridra* for delayed leaf senescence using *PSAG12-IPT*. MS Thesis, Univ. of Florida, Gainesville.

Fry, J. and B. Huang. 2004. Applied turfgrass science and physiology. Wiley, Hoboken, NJ.

Fu-Beder, A., A. Watertinger, W. Hartung, E.D. Schulze, and H. Heilmeyer. 1992. Cytokinins in the xylem sap of desert-grown almond (*Prunus dulcis*) (Miller) D.A. Webb trees: Daily courses and their possible interactions with abscisic acid and leaf conductance. New Phytol. 122:45–52.

Gan, S.S. and R.M. Amasino. 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270:1986–1988.

Goicoechea, N., M.C. Antolin, and M. Sanchez-Diaz. 2006. Gas exchange is related to the hormone balance in mycorrhizal or nitrogen-fixing alfalfa subjected to drought. Physiol. Plant. 100:989–997.

Havlova, M., P.I. Dobrev, V. Motyka, H. Storchova, J. Libus, J. Dobra, J. Malbeck, A. Gaudinova, and R. Vankova. 2008. The role of cytokinins in responses to water deficit in tobacco plants over-expressing trans-zeatin *O*-glucosyltransferase gene under 35S or *SAG12* promoters. Plant Cell Environ. 31:341–353.

Hewelt, A., E. Prinsen, J. Schell, H. Van Onckelen, and T. Schmulling. 1999. Regulation of flooding tolerance of *Arabidopsis* by cytokinin. J. Expt. Bot. 50:1397–1407.

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

Huynh, L.N., T. Van Toai, J. Streeter, and G. Banowetz. 2005. Regulation of flooding tolerance of *SAG12-IPT* arabidopsis plants by cytokinin. J. Expt. Bot. 56:1397–1407.

Jordi, W., A. Schapendonk, E. Davelaar, G.M. Stoopen, C.S. Pot, R. De Visser, J.H.A. Van Rhijn, J.B. Power, and M.R. Davey. 2001. Effects of *PSAG12-IPT* gene expression on development and senescence in transgenic lettuce. Plant Physiol. 127:505–516.

Karcher, D.E., M.D. Richardson, K. Hignight, and D. Rush. 2008. Drought tolerance of tall fescue populations selected for high root/shoot ratios and summer survival. Crop Sci. 48:771–777.

Khan, A.A. 1981. Effect of leaf position and plant age on the translocation of 14C-assimilates in onion. J. Agr. Sci. 96:451–455.

Klee, H.J. 1994. Transgenic plants and cytokinin biology, p. 289–293. In: W.S. Mok and M.S. Mok (eds.). Cytokinins: Chemistry, activity, and function. CRC Press, Boca Raton, FL.

Kovtun, Y., W.L. Chiu, G. Tena, and J. She. 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc. Natl. Acad. Sci. USA 97:2940–2945.

Kramer, P.J. and J.S. Boyer. 1995. Water relations of plants and soils. Academic Press, New York.

Larkin, P.J. and W.R. Scowcroft. 1981. Solomacial variation: A novel source of variability from cell cultures for plant improvement. Appl. Genet. 60:197–214.

Letham, D.S. and L.M.S. Paimi. 1983. The biosynthesis and metabolism of cytokinins. Annu. Rev. Plant Physiol. 34:163–197.

Liu, X. and B. Huang. 2002. Cytokinin effects on creeping bentgrass responses to heat stress II. Antioxidant enzyme activities and lipid peroxidation. Crop Sci. 42:466–472.

Liu, X. and B. Huang. 2005. Root physiological factors involved in cool-season grass response to high soil temperature. Environ. Exp. Bot. 53:233–245.

Liu, X., B. Huang, and G. Banowetz. 2002. Cytokinin effects on creeping bentgrass responses to heat stress: I. Shoot and root growth. Crop Sci. 42:457–465.

Luo, Y.Y., T.J. Gianfagna, H.W. Janes, B. Huang, Z. Wang, and J. Xing. 2005. Expression of the ipt gene with the AGPase s1 promoter in tomato results in unbranched roots and delayed leaf senescence. Plant Growth Regulat. 47:47–56.

Marrion-Poll, A. and J. Leung. 2006. Abscisic acid synthesis, metabolism, and signal transduction, p. 1–35. In: P. Hedden and T.G. Thomas (eds.). Plant hormone signaling. Blackwell Publishing, Oxford, UK.

McCabe, M.S., L.C. Garratt, F. Scheppers, W.J.R.M. Jordi, G.M. Stoopen, E. Davelaar, J. Hans, A. van Rijn, J.B. Power, and M.R. Davey. 2001. Effects of *PSAG12-IPT* gene expression on development and senescence in transgenic lettuce. Plant Physiol. 127:505–516.

Mok, D.W. and M.C. Mok. 1994. Cytokinins: Chemistry, activity, and function. CRC Press, Boca Raton, FL.

Mok, D.W. and M.C. Mok. 2001. Cytokinin metabolism and action. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52:89–118.

Morris, R.O. 1995. Genes specifying auxin and cytokinin biosynthesis in prokaryotes, p. 318–339. In: P.J. Davies (ed.). Plant hormones, physiology, biochemistry, and molecular biology. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Naqvi, S.S.M. 1995. Plant/crop hormones under stressful conditions, p. 645–660. In: M. Pessarakli (ed.). Handbook of plant and crop physiology. Marcel Dekker, New York.

Okamoto, M., K. Tatematsu, A. Matsui, T. Morosawa, J. Ishida, M. Tanaka, T. Endo, Y. Mochizuki, T. Toyoda, Y. Kamiya, K. Shinozaki, E. Nambara, and M. Seki. 2010. Genome-wide analysis of endogenous abscisic acid-mediated transcription in dry and imibed seeds of arabidopsis using tiling arrays. Plant J. 62:39–51.

Pospisilova, J., H. Synkova, and J. Rulcova. 2000. Cytokinins and water stress. Biol. Plant. 43:321–328.

Pospisilova, J., M. Vagner, J. Malbeck, A. Travnickola, and P. Batkova. 2005. Interactions between abscisic acid and cytokinins during water stress and subsequent rehydration. Biol. Plant. 49:533–540.

Rivero, R.M., M. Kojima, A. Gepstein, H. Sakakibara, R. Mittler, S. Gepstein, and E. Blumwald. 2007. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proc. Natl. Acad. Sci. USA 104:19631–19636.

Rivero, R.M., V. Shulaev, and E. Blumwald. 2009. Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. Plant Physiol. 150:1530–1540.
Sakuma, Y., K. Maruyama, F. Qin, Y. Osakabe, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2006. Dual function of an arabidopsis transcription factor DREB2A in water-stress-responsive and heat-stress-responsive gene expression. Proc. Natl. Acad. Sci. USA 103:18822–18827.

Schnablova, T., H. Synkova, A. Vicankova, L. Burketova, J. Eder, and M. Cvikrova. 2006. Transgenic ipt tobacco overproducing cytokinins overaccumulates phenolic compounds during in vitro growth. Plant Physiol. Biochem. 44:526–534.

Setter, T.L., B.A. Flannigan, and J. Melkonian. 2001. Loss of kernel set due to water deficit and shade in maize: Carbohydrate supplies, abscisic acid, and cytokinins. Crop Sci. 41:1530–1540.

Takahashi, T. and Y. Komeda. 1989. Characterization of two genes encoding small heat-shock proteins in arabidopsis. J. Mol. Genet. 219:365–372.

Topp, G.C. 1980. Electromagnetic determination of soil water content: Measurements in coaxial transmission lines. Water Resour. Res. 16:574–582.

Turgeon, A.J. 2008. Turfgrass management. 8th ed. Pearson Prentice Hall, Upper Saddle River, NJ.

Xing, J., Y. Xu, J. Tian, T. Gianfagna, and B. Huang. 2010. Transformation of a perennial grass species with ipt gene controlling cytokinin synthesis associated with suppression of shade or heat-induced leaf senescence. J. Amer. Soc. Hort. Sci. 134:602–609.

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

Van Loven, K., S. Beinsberger, R. Valcke, H. Van Onckelen, and H. Clijsters. 1993. Morphometric analysis of the growth of Phsp70-ipt transgenic tobacco plants. J. Expt. Bot. 44:1671–1678.

Verdonk, J.C., K. Shibuya, H.M. Loucas, T.A. Colquhoun, B.A. Underwood, and D.G. Clark. 2008. Flower-specific expression of the agrobacterium isopentenyltransferase gene results in radial expansion of floral organs in Petunia hybrida. Plant Biotechnol. J. 6:694–701.

Volaire, F., H. Thomas, N. Bertagne, E. Bourgeois, M.F. Gautier, and F. Lelievre. 1998. Survival and recovery of perennial forage grasses under prolonged Mediterranean drought: Water status, solute accumulation, abscisic acid concentration and accumulation of dehydrin transcripts in bases of immature leaves. New Phytol. 140:451–460.

Wang, Z., B. Huang, and Q. Xu. 2003. Effects of abscisic acid on drought response of kentucky bluegrass. J. Amer. Soc. Hort. Sci. 128:36–41.

Yoshida, K. and A. Shinmyo. 2000. Transgene expression systems in plant, a natural bioreactor. J. Biosci. Bioeng. 90:353–362.

Zhang, X. and E.H. Ervin. 2004. Cytokinin-containing seaweed and humic acid extracts associated with creeping bentgrass leaf cytokinins and drought resistance. Crop Sci. 44:1737–1745.

Zhang, J., T. Van Toai, L. Huynh, and J. Preiszner. 2000. Development of flooding-tolerant arabidopsis by autoregulated cytokinin production. Mol. Breed. 6:135–144.

Zhang, Y., C. Liang, Y. Xu, T. Gianfagna, and B. Huang. 2010. Effects of ipt gene expression on leaf senescence induced by nitrogen or phosphorus deficiency in creeping bentgrass. J. Amer. Hort. Sci. 135:108–115.