Suppression of Shade- or Heat-induced Leaf Senescence in Creeping Bentgrass through Transformation with the *ipt* Gene for Cytokinin Synthesis

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ABSTRACT. Cytokinins have been associated with delaying or suppressing leaf senescence in plants. The objectives of this study were to determine whether the expression of the *ipt* gene that encodes adenine isopentenyltransferase would delay leaf senescence induced by shade or heat stress in a perennial grass species. Creeping bentgrass (*Agrostis stolonifera* cv. Penncross) was transformed with *ipt* isolated from agrobacterium (*Agrobacterium tumefaciens*) using two gene constructs (*SAG12-ipt* and *HSP18-ipt*) designed to activate cytokinin synthesis during shade or heat stress. Whole plants of nine *SAG12-ipt* transgenic lines and the nontransgenic control plants were incubated in darkness at 20 °C for 20 days. Chlorophyll content of all transgenic lines and the control line decreased after dark treatment, but the decline was less pronounced in transgenic lines. All transgenic lines had higher isopentenyladenine (iP/iPA) content than the control line after 20 days of treatment. In six of the transgenic lines, iP/iPA content remained the same or higher after dark treatment. Whole plants of nine *HSP18-ipt* transgenic lines and the control plants were incubated at 35 °C for 7 days. Chlorophyll and iP/iPA content declined in the control plants, but the nine transgenic lines had a significantly higher concentration of iP/iPA and were able to maintain chlorophyll content at the prestress level. Our results suggest that expression of *SAG12-ipt* or *HSP18-ipt* in creeping bentgrass resulted in increases in cytokinin production, which may have led to the delay and suppression of leaf senescence induced by shade or heat stress.

Naturally occurring and environmentally induced leaf senescence limits whole-plant photosynthetic capacity and plant productivity, as well as the aesthetic value of horticultural plants. Leaf senescence is characterized by yellowing or chlorosis of leaves as chlorophyll and other cellular components (e.g., proteins and nucleic acids) are degraded during natural or stress-induced leaf aging. Cytokinins (CK) have been well known for delaying leaf senescence, and in some cases, reversing this process (Gan and Amasino, 1996). Moreover, there is generally an inverse correlation between CK content and the severity of leaf senescence (Hare et al., 1997).

One approach to prevent or delay leaf senescence is to increase CK in leaves through the application of products containing CK, or to overexpress genes controlling CK synthesis through genetic transformation. Transgenic plants with modified endogenous CK production have recently been used to study the involvement of CK in delaying leaf senescence, using the gene encoding adenine isopentenyltransferase (*ipt*) isolated from agrobacterium. The *ipt* gene catalyzes the key step in de novo CK biosynthesis: the formation of N⁶-(Δ^2 -isopentenyl) adenosine-5'-monophosphate from Δ^2 -isopentenyl pyrophosphate and s-adenosine-5'-monophosphate (Medford et al., 1989). This gene has been introduced into various plant species, mostly dicotyledonous plants, such as tobacco (*Nicotiana tabacum*)

(Gan and Amasino, 1996), cauliflower (*Brassica oleracea* var. *botrytis*) (Nguyen et al., 1998), lettuce (*Lactuca sativa*) (McCabe et al., 1998; McCabe et al., 2001), arabidopsis (*Arabidopsis thaliana*) (Huynh et al., 2005), petunia (*Petunia $\times$ hybrida*) (Chang et al., 2003; Clark et al., 2004; Khodakovskaya et al., 2005), chrysanthemum (*Dendranthema $\times$ grandiflorum*) (Khodakovskaya et al., 2005), and tomato (*Solanum lycopersicum*) (Luo et al., 2005), and in a limited number of monocot plant species such as tall fescue (*Festuca arundinacea*) (Hu et al., 2005) and wheat (*Triticum aestivum*) (Sykorova et al., 2008). Most studies have confirmed that increases in endogenous production of CK delayed leaf senescence. However, some plants transformed with a high-expression *ipt* gene construct driven by constitutive promoters exhibit phenotypic signs of hormone surplus and growth abnormalities, such as dwarfism and limited root growth, due to overproduction of CK (Hewelt et al., 1994; Schmülling et al., 1989; Smigocki, 1991).

The expression of the *ipt* gene controlled by regulatable or inducible promoters prevents the problems associated with the overproduction of CK in transgenic plants with constitutive promoters. A senescence-activated promoter, *SAG12*, was isolated from arabidopsis to drive *ipt* expression to delay leaf senescence (Gan and Amasino, 1995). The *SAG12-ipt* construct has an autoregulatory feature: the transcription of *ipt* is activated by *SAG12* at the onset of leaf senescence, leading to the production of functional enzyme and CK production, which in turn delays senescence; when there are no longer senescence signals, the *SAG12* promoter attenuates *ipt* transcription and subsequent enzyme production, thus providing autoregulatory control of CK synthesis (Gan and Amasino, 1995, 1996). Similarly, Rivero et al. (2007) used the promoter from a senescence-associated

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receptor protein kinase gene (*SARK*) as a promoter for *ipt*. Expression of *SARK-ipt* resulted in delayed drought-induced leaf senescence in tobacco. Another class of commonly used promoters are those regulated by heat shock. In response to elevated temperatures, the synthesis of a family of protective proteins, named heat shock proteins (HSP), is induced. Transgenic plants of various species using heat shock promoters to control *ipt* gene transcription (*HSP-ipt*) have been created that increase CK synthesis and reduce leaf senescence under high temperature stress (Medford et al., 1989; Schmülling et al., 1989; Smart et al., 1991; Smigocki, 1991; Van Loven et al., 1993).

Leaf senescence is a major concern in turfgrass management because it not only negatively affects plant growth, but also the aesthetic turf quality. Turf quality often declines due to leaf senescence induced under environmental stresses such as shading and high temperature (Huang, 2004; Koh et al., 2003). Preventing or delaying leaf senescence is an effective approach to improve turf quality, especially in unfavorable environments. However, how *ipt* controlling CK synthesis may affect shade- or heat-induced leaf senescence has not been examined in turfgrass species. Using an agrobacterium-mediated transformation technique, reporter genes or other target genes have been successfully transferred into several turfgrass species, including creeping bentgrass (Dong and Qu, 2005; Yu et al., 2000). With an aim to investigate whether activation of the *ipt* gene would delay leaf senescence in cool-season grass species exposed to shade or heat stress, we transformed cool-season creeping bentgrass, a widely used turfgrass species, using an agrobacterium-mediated transformation technique with two constructs: *SAG12-ipt* and *HSP18-ipt*. The expression pattern of *SAG12-ipt* and *HSP18-ipt* induced by shade or heat stress was examined, and leaf senescence and CK production associated with *ipt* gene expression were evaluated.

Materials and Methods

TISSUE CULTURE AND PLANT REGENERATION. Stolons of creeping bentgrass (cv. Penncross) were collected from a single plant for tissue culture to generate transgenic plants with identical genetic background. Stolons were cut to pieces and treated with 95% alcohol for 1 min, followed by 15% household bleach (6.15% sodium hypochlorite) for 5 min, and washed with sterile water (five times) before placing in tissue culture medium. Calluses were produced in darkness at 24 °C on a tissue culture medium containing 4.3 g Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962), 500 mg of casein hydrolysate, 100 mg of myoinositol, 6.6 mg of dicamba, 2.5 mg of benzyladenine (BA) (Sigma-Aldrich, St. Louis), 30 g of sucrose, and 2 g of Gell-Gro (*Pseudomonas elodea*; ICN Bio-medicals, Irvine, CA) in 1 L of water at pH 5.7. Calluses were formed and ready for transformation after 1 month of culture.

Plantlets were generated from calluses by transferring to the regeneration medium [RM (4.3 g of MS base, 100 mg of myoinositol, 4 mg of BA, 20 g of sucrose, and 2.0 g of Gell-Gro in 1 L water at pH 5.8)] in a controlled chamber at 24 °C, with a 12-h photoperiod, and 85 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux (PPF; 400–700 nm). Emerging

plantlets were subcultured at 2-week intervals. Seedlings with about five tillers each were transplanted into containers filled with soil and were kept in a greenhouse with a mean day/night temperature of 24/18 °C, with a 12/12-h light/dark photoperiod, and light intensity ranging from 500 to 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

PLASMID CONSTRUCTION. Two inducible promoters from arabidopsis, *SAG12* and *HSP18*, were used in the plasmid constructs to introduce the *ipt* gene into creeping bentgrass. The senescence-specific *SAG12* promoter was used to drive the expression of the *ipt* gene at the onset of leaf senescence (Gan and Amasino, 1995). *HSP18*, a heat-inducible promoter, drives the expression of *ipt* when exposed to heat stress (Takahashi and Komeda, 1989). The *ipt* gene is from the Ti plasmid of agrobacterium. The construct pCAMBIA1300-*SAG12-ipt* was created from pSG516 (provided by R. Amasino, University of Wisconsin) and pCAMBIA 1300 (CAMBIA, Canberra, Australia), a binary plasmid containing the gene for hygromycin resistance. pSG516 contains the *SAG12* promoter, *ipt* gene, and *NOS* ending region. pSG516 was cut with *SpeI* and the target fragment was inserted into pCAMBIA 1300 at the *XbaI* site to create pCAMBIA1300-*SAG12-ipt* (Fig. 1A).

The construct pCAMBIA1301-*HSP18-ipt-GUS* was created from pUC-*HSP18*, pSG516, and pCAMBIA 1301 (GenBank accession no. 234297), a binary plasmid containing the genes for hygromycin and β -glucuronidase (*GUS*). pUC-*HSP18* was cut by *NotI* and *NcoI* to release the *HSP18* promoter (0.7 kb). pSG516 (5.88 kb) was also cut with *NotI* and *NcoI* to release the *SAG12* promoter (2.2 kb). The 3.68-kb fragment containing *ipt-NOS* was ligated to the *HSP18* promoter to create a new plasmid pJP101 (4.38 kb) containing *HSP18-ipt-NOS*. pJP101 was cut with *HindIII* and *XbaI* and the *HSP18-ipt-NOS* fragment was inserted into the multicloning site of pCAMBIA1301 to create pCAMBIA1301-*HSP18-ipt-GUS* plasmid (Fig. 1B). Control lines were created using the empty vector.

AGROBACTERIUM-MEDIATED CREEPING BENTGRASS TRANSFORMATION. Agrobacterium LBA 4404 was transformed by electroporation at 2400 mV using the constructs described above and empty vector control plasmids, and was grown at 28 °C for 2 d. Agrobacterium LBA 4404 carrying the plasmid constructs was grown in Lysogeny broth (LB) medium supplemented with 50 $\text{mg}\cdot\text{L}^{-1}$ kanamycin, 50 $\text{mg}\cdot\text{L}^{-1}$ spectinomycin, and 50 μM acetosyringone on a platform shaker (28 °C, 200 rpm, and 48 h). Agrobacterium culture was collected by centrifugation (at 2236 g_n for 10 min) and resuspended to OD₆₀₀ of 0.2 in a liquid coculture medium [LCM, a Phytigel-free callus induction medium with addition of 50 μM acetosyringone and modified carbohydrate source (10 $\text{g}\cdot\text{L}^{-1}$ sucrose and 20 $\text{g}\cdot\text{L}^{-1}$ glucose)]. Embryogenic calluses, $\approx$ 40-d-old and pretreated with 50 μM acetosyringone, were cocultured with agrobacterium cell suspension for 30 min at room temperature. The agrobacterium-infected

Fig. 1. Schematic diagram of plasmid constructs. (A) pCAMBIA1300-*SAG12-ipt* and (B) pCAMBIA1301-*HSP18-ipt-GUS*; LB = T-DNA left border, RB = T-DNA right border, *SAG12* = arabidopsis senescence-activated promoter 12, *ipt* = isopentenyladenine transferase gene, CaMV 35S = cauliflower mosaic virus 35S promoter, *Hph* = hygromycin phosphotransferase gene, CaMV 35S 3' = 3' untranscribed region of the nopaline synthase gene, *HSP18* = arabidopsis heat shock 18 promoter, *Gus* = β -glucuronidase (*uidA*) gene.

calluses were cocultured on tissue culture medium with the addition of cefotaxime (250 mg·L⁻¹) for 1 week and were then transferred to a selection medium with the addition of hygromycin (175 mg·L⁻¹). Healthy calluses were then moved to regeneration medium. Regenerated seedlings were grown on media containing hygromycin (175 mg·L⁻¹) and those that survived antibiotic screening were considered to be hyg B-resistant plants and were selected for further analysis.

POLYMERASE CHAIN REACTION (PCR) ANALYSIS. Transformation was confirmed using PCR analysis. One 2-cm-long fresh grass leaf was collected from each line of the transgenic plants. The leaves were treated with 100 µL of 0.4 M NaOH at 100 °C for 5 min, followed by 100 µL of 0.5 M Tris-HCl at pH of 8.0 and 100 µL of 0.4 M HCl. A 2-µL aliquot of the above solution was used as a template for PCR. A 50-µL PCR reaction solution was prepared, containing 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.4 mM dNTP, 2 µL of DNA template, 1 unit of Taq DNA polymerase, and 0.2 µM primers (5'-GACCTGCATCTAA TTTTCGGTCCAAC-3'/5'-GGGGTGCAACATCTGCTTAAC TCT-3'). The *ipt* gene-specific primers were designed based on the 723-bp agrobacterium *ipt* complete sequence [gi|10955016:7864-8586] using primer3 (Invitrogen, Carlsbad, CA). Reaction conditions were: 94 °C for 1 min (1 cycle), then 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min (35 cycles), 72 °C for 10 min, and were then kept at room temperature to generate a 432-bp fragment

GUS STAINING. Histochemical staining for GUS activity was performed following the protocol described in Jefferson et al. (1987). The solution contained 0.01 M NaH₂PO₄, 0.005 M EDTA, 0.05 mM K₃FeCN₆ and K₄FeCN₆, and 0.1 mg·mL⁻¹ X glucuronide. Root tissues were incubated in GUS staining solution for 6 h or overnight at 37 °C and washed with water. Leaf tissues were removed with 75% ethanol for 3 h followed by 95% ethanol for 3 h and 100% ethanol overnight. After bleaching, tissue was washed in water for 1 h.

SOUTHERN BLOT ANALYSIS OF TRANSGENE COPY NUMBERS. Selected transgenic lines were tested for copy number of *ipt*. Genomic DNA was extracted from leaf tissues using the maize (*Zea mays*) miniprep method (Dellaporta et al., 1983). The genomic size of creeping bentgrass is 2769 Mbp per cell. The construct was about 13.5 kb in size. The ratio of genomic size to construct size was about 205000:1. For Southern blot analysis, 5 µg of genomic DNA, 0.025 ng of construct plasmid DNA (1 copy), 0.050 ng of construct plasmid DNA (2 copy), 0.075 ng of construct plasmid DNA (3 copy), 0.100 ng of construct plasmid DNA (4 copy), 0.125 ng of construct plasmid DNA (5 copy), and 0.150 ng of construct plasmid DNA (6 copy) were loaded onto Hybond N⁺ membranes (GE Healthcare, Piscataway, NJ). The coding region of *ipt* gene was radioactively labeled with α-³²P-dCTP by using the random primer labeling system (Promega, Madison, WI) and was purified by Micro-Spin™ G-50 Columns (GE Healthcare). Prehybridization and hybridization were carried out in 50% (v/v) formamide, 5× SSPE, 10× Denhardt's, 1% SDS, and herring sperm DNA (300 mg·L⁻¹) in a total volume of 15 mL at 42 °C overnight. Washes were performed in 2× SSPE and 1% SDS for 25 min at room temperature and in three additional steps with preheated 0.2× SSPE and 0.2% SDS at 65 °C for 5 to 20 min. The membranes were exposed to X-Ray film at -70 °C for 3 d.

RNA ISOLATION AND NORTHERN BLOT. RNA was isolated by a miniprep procedure (Ausubel et al., 1995). Briefly, about 3 g of leaves were ground in liquid N₂ in a mortar with pestle. The

ground tissue was added to a tube containing 5 mL of water-saturated phenol and 5 mL of preheated (80 °C) RNA extraction buffer (200 mM Tris-HCl, pH 8.0, 100 mM LiCl, 20 mM EDTA, and 1% SDS) and was vortexed. One-half volume chloroform: isoamyl alcohol (24:1) was added and the sample was vortexed again. After centrifugation, the upper aqueous phase was removed to a new tube and 1 volume of 4 M LiCl was added, mixed well, and placed at -70 °C for 1 h. The sample was centrifuged for 15 min. The pellet was then washed in DEPC-treated 70% ethanol, air dried, and resuspended in 500 µL of DEPC-treated water.

RNA samples (10 µg) were separated on 1.2% formaldehyde agarose gels and transferred to membranes (Magnacharge nylon Membrane; BioWorld, Dublin, OH). RNA blots were hybridized with the probe containing the coding region (520 bp) of the *ipt* gene that was labeled by a random primer labeling kit (Prime-a-Gene kit, Promega) with ³²P-dCTP. Northern blots were hybridized at 65 °C for 24 h in 2× SSPE, SDS, PEG 8000, and heparin (Sigma-Aldrich). Denatured herring sperm DNA (0.2 mg·mL⁻¹) was added to the denatured, labeled probe and the solution was diluted to a final concentration of 106 counts per minute per milliliter for hybridization. RNA membranes were washed twice in 2× SSC and 0.1% SDS for 40 min, and twice in 0.5× SSC and 0.1% SDS for 40 min. The membranes were exposed to X-Ray film at -70 °C for 3 to 7 d.

CHARACTERIZATION OF EXPRESSION PATTERN OF *HSP18-IPT* AND *SAG12-IPT*. The expression pattern of the *HSP18* promoter in creeping bentgrass in response to increasing temperatures and treatment durations was examined. For time courses, excised leaves or whole plants of *HSP18-ipt* transgenic lines were exposed to 35 °C in a growth chamber for 2, 4, and 12 h. Leaf samples were collected for Northern blot analysis. For temperature courses, excised leaves or whole plants of *HSP18-ipt* transgenic lines were exposed to 20, 25, 30, 35, and 40 °C for 2 h in growth chambers, and leaf samples were then collected for Northern blot analysis as described above.

The expression pattern of *SAG12-ipt* during dark- or heat-induced leaf senescence was evaluated for selected transgenic lines. Whole plants were exposed to light (500 µmol·m⁻²·s⁻¹) at 20 °C (control), dark at 20 °C (shade), or light at 35 °C (heat stress) for 7 d in a growth chamber. Leaf samples were collected for Northern blot analysis as described above.

EVALUATION OF LEAF SENESCENCE AND QUANTIFICATION OF CK PRODUCTION. Nine transgenic lines of *SAG12-ipt* and *HSP18-ipt* transformants that exhibited desirable turf phenotypic traits (fine leaf texture and uniform green leaves) were selected for the physiological evaluation.

Detached leaves and intact plants of *SAG12-ipt* or *HSP18-ipt* transgenic plants were used to determine whether transgenic plants exhibit delayed leaf senescence induced by dark or heat stress in comparison with the control line (transformed with the empty vector without *ipt*). Leaf senescence was evaluated by measuring the chlorophyll content of excised or intact leaves exposed to the conditions, inducing senescence. Excised leaves were incubated in darkness in half-strength Hoagland's nutrient solution; with 10 µM transzeatin riboside (ZR; Sigma-Aldrich) added to the incubation solution of the control line. Northern blot and CK content were analyzed for whole plants exposed to dark or heat stress to examine gene expression and CK content during dark- or heat-induced leaf senescence. Chlorophyll content was calculated based on the absorbance at 663 and 645 nm using the formulas described by Arnon (1949). A separate set

of leaf samples were taken from the same plant at the same time of sampling for chlorophyll extraction, and fresh weight was measured immediately. Leaf chlorophyll content was expressed as milligrams per gram of fresh weight.

Two major forms of CK, transzeatin/zeatin riboside (Z/ZR) and isopentenyladenine/adenosine (iP/iPA), were quantified by an indirect competitive enzyme-linked immunoabsorbent assay (ELISA). Extraction and quantification of hormones followed the method described by Setter et al. (2001) with some modifications (Wang et al., 2003). Briefly, samples were extracted in 80% (v/v) methanol and were isolated with reverse phase C₁₈ columns. Hydrophilic contaminants were washed out with 200 μ L of 20% solvent [20% methanol, 80% aqueous TEA (10 mM triethylamine, pH 3.5)]. The CK-containing fraction was eluted using 200 μ L of 30% solvent [30% methanol, 70% aqueous triethylamine (TEA)]. An indirect competitive ELISA was used for quantification of Z/ZR and iP/iPA as previously described by Setter et al. (2001). Monoclonal antibodies against Z/ZR and iP/iPA (Agdia, Elkhart, IN) were originally developed by Eberle et al. (1986).

TREATMENTS. For the dark treatments, the second fully expanded leaves from the top of five tillers in each plant of the transgenic line were incubated in half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) in petri dishes placed in darkness at 25 °C for 30 d; whole plants grown in soil-filled pots were exposed to darkness in a growth chamber at 20 °C for 20 d. For heat treatment, whole plants were exposed to 35 °C for 7 d and were watered daily to prevent water deficit.

STATISTICAL ANALYSIS. All physiological measurements and cytokinin analyses were performed in four replicated samples (plants). For light, dark, or temperature treatment, four replicated plants were examined for each treatment and each transgenic line. Data were analyzed using analysis of variance to determine treatment effects and difference between the control line and transgenic plants. The differences among lines and between treatments for a given line were separated using Fisher's protected least significant difference test at $P = 0.05$.

Results

CONFIRMATION OF *IPT* TRANSFORMATION BY PCR AND GUS STAINING. A total of 142 *SAG12-ipt* and 138 *HSP18-ipt* putatively transformed plants were examined for *ipt* expression using PCR analysis. Over 95% of the plants exhibited positive PCR. Figure 2 illustrates *ipt* expression in nine transgenic lines containing *HSP18-ipt* and nine transgenic lines containing *SAG12-ipt*. GUS staining of roots was also performed to confirm plant transformation. Roots of transgenic plants were stained blue while nontransgenic plants had no blue staining in their roots (Fig. 2). Two selected transgenic lines were tested for transgene copy number. The transgene was present in single copy in *SAG12-ipt* and *HSP18-ipt* lines, as indicated by the quantity of DNA present in Southern blot analysis (Fig. 3).

EXPRESSION OF *IPT* IN *SAG12-IPT* AND *HSP18-IPT* PLANTS IN RESPONSE TO DARKNESS OR HEAT STRESS. Thirty-three lines of *SAG12-ipt* and 35 *HSP18-ipt* plants with desirable turf phenotype under nonstressed conditions were selected and examined for gene expression under darkness or heat stress at 35 °C. Northern blot analysis did not detect *SAG12-ipt* expression in plants exposed to normal light and temperature (20 °C), but revealed that among the 33 *SAG12-ipt* transgenic plants, 58% showed *ipt* expression when excised leaves were exposed to

Fig. 2. Confirmation of transformation with GUS staining in the empty vector control line (A and B) and four *HSP18-ipt* lines (C–F) and PCR analysis in nine representative *SAG12-ipt* line (G) and nine representative *HSP18-ipt* lines (H) of creeping bentgrass. M = marker, EV = plants transformed with empty vector, PL = plasmid containing the *ipt* gene.

Fig. 3. Analysis of transgene copy number in two representative transgenic lines (*SAG12-ipt* transgenic line S37 and *HSP18-ipt* transgenic line H13) of creeping bentgrass. G1 = total DNA equal to one copy of creeping bentgrass total DNA, G2 = total DNA equal to two copies of creeping bentgrass total DNA; 1, 2, 3, 4, 5, and 6 = plasmid DNA equal to one, two, three, four, five, and six copies of creeping bentgrass total DNA, respectively.

darkness for 20 d (Fig. 4A). For the 35 *HSP18-ipt* transgenic plants, 63% showed *ipt* transcript when excised and subjected to heat stress at 35 °C for 7 d (Fig. 4B). Whole plants of two *SAG12-ipt* lines, S37 and S43, were also exposed to darkness for 20 d or 35 °C for 7 d. Darkness and heat stress activated the expression of *SAG12-ipt*, but the expression of *ipt* was more strongly induced by darkness than heat stress at 35 °C for both *SAG12-ipt* lines (Fig. 4C).

The expression patterns of *HSP18-ipt* in whole plants exposed to different temperatures for different durations were examined in the transgenic line H31. Northern blot analysis of

H31 plants treated at each temperature of 20, 25, 30, 35, and 40 °C for 2 h showed that *ipt* expression was strongly induced when temperature was elevated to 35 and 40 °C (Fig. 5A). With heat treatment duration, expression of *ipt* was detected at 2 h after H31 plants were exposed to 35 °C, and the expression level increased with treatment duration up to 12 h (Fig. 5B).

EVALUATION OF LEAF SENESCENCE AND CYTOKININ PRODUCTION IN *SAG12-IPT* PLANTS EXPOSED TO DARKNESS. An excised leaf bioassay was performed to determine the response of nine *SAG12-ipt* transgenic lines to dark-induced leaf senescence. The third fully expanded leaves were clipped from each of the *SAG12-ipt* transgenic lines and the control line. Leaves were incubated in nutrient solution in the dark at 20 °C in petri dishes for 30 d. After 14 d in darkness, the control plants turned yellow while the transgenic leaves remained green; after 30 d in dark, the control leaves became completely chlorotic while the transgenic leaves still had some green spots (Fig. 6). Leaf chlorophyll content of all nine transgenic lines was significantly higher than control plants at 14 d of dark treatment, with two transgenic lines maintaining chlorophyll content twice that of the control and similar to the control leaves incubated with CK (Fig. 7).

Whole plants of nine *SAG12-ipt* transgenic lines were also incubated in darkness in growth chambers at 20 °C for 20 d, and

Fig. 4. Northern confirmation of *ipt* gene expression among different transgenic lines of creeping bentgrass. (A) *SAG12-ipt* plants exposed to dark at 20 °C for 20 d. (B) *HSP18-ipt* plants exposed to 35 °C for 7 d. (C) *SAG12-ipt* line S37 and S43 exposed to dark at 20 °C or heat stress at 35 °C. Each lane was loaded with 10 µg of RNA for each sample.

Fig. 5. Temperature and time course changes in *ipt* gene expression of a *HSP18-ipt* transgenic line (H31) of creeping bentgrass. (A) Temperature course study of H31 treated for 2 h at 20, 25, 30, 35, and 40 °C. (B) Time course study of H31 treated at 35 °C for 0, 2, 4, 8, 12, and 24 h. Each lane was loaded with 10 µg of RNA for each sample.

Fig. 6. Comparison of morphological differences between detached mature leaves of an empty vector control line (A, C, and E) and a *SAG12-ipt* transgenic line (S12) (B, D, and F) of creeping bentgrass. Leaves were incubated in nutrient solution in the dark at 25 °C in petri dishes for 30 d. (A and B) Leaves from the control line and *SAG12-ipt* line before dark treatment. After 2 weeks, leaves from the control line (C) turned yellow, while the *SAG12-ipt* transgenic leaves were still fresh green (D). After 1 month, leaves from the control line were all white-yellow (E), while the transgenic leaves still had some green sections (F).

Fig. 7. Chlorophyll content of detached leaves from nine *SAG12-ipt* transgenic lines and control line of creeping bentgrass before (Pre-dark) and after incubation in the dark at 20 °C for 14 d. Control without (Control) or with ZR (Control + 10 µM ZR). Columns marked with the same letters were not significantly different based on LSD test at $P = 0.05$.

chlorophyll content and CK content of leaves were determined. Chlorophyll content of all nine transgenic lines and the control decreased after 21 d of dark treatment, but the decline was less pronounced in all nine transgenic lines. Chlorophyll content of transgenic lines decreased an average of 23.1%, whereas chlorophyll content of the control line declined 62.9% during dark treatment (Fig. 8). In six of the transgenic lines, the iP/iPA content remained the same or higher after dark treatment, whereas a significant reduction in iP/iPA content occurred in the control line and in two transgenic lines (S15 and S48) after 20 d of dark treatment; nevertheless, the reduction was less in the two transgenic lines (19.1% and 29.9%) than in the control line (56.4%) (Fig. 9). All nine transgenic lines had higher iP/iPA content than the control line at 20 d of treatment. No significant differences in Z/ZR content were detected between the control and transgenic lines during dark or heat treatment (data not shown).

HEAT STRESS RESPONSES OF *HSP18-IPT* TRANSGENIC PLANTS.

When whole plants were incubated at 35 °C for 7 d, chlorophyll content declined 38.2% in the control plants, but all nine transgenic plants were able to maintain chlorophyll content at the initial level (Fig. 10). All nine transgenic lines also had significantly higher chlorophyll content than the control line under heat stress, although no significant differences in chlorophyll content were detected at 20 °C. The effects of heat stress and differences between the control and transgenic lines for iP/iPA content followed the same pattern as chlorophyll content (Fig. 11). The iP/iPA content of the control line declined 52.5%, whereas the transgenic plants maintained iP/iPA production and had higher iP/iPA content than the control plants under heat stress.

Discussion

Our study demonstrated that the transformation of *SAG12-ipt* resulted in the suppression of leaf senescence induced by dark or heat stress without the morphological abnormalities usually observed with constitutive promoters due to the autoregulation of CK synthesis (Gan and Amasino, 1995). The suppression of dark-induced leaf senescence, as demonstrated by the maintenance of higher chlorophyll content in the transgenic lines com-

pared with the control line, could be associated with the greater production of iP/iPA in the transgenic plants. Luo et al. (2005) also found excised tomato leaf senescence to be delayed when *ipt* was ligated to the AGPase *S1* promoter, which is active in the guard cells of the leaves. Li et al. (2004) found a significant delay in whole plant leaf senescence of perennial ryegrass (*Lolium perenne*) transformed by microprojectile bombardment with *SEE-ipt*. This delay in leaf senescence of grasses could have important economic consequences, especially after

Fig. 8. Leaf chlorophyll content of whole plants grown of creeping bentgrass under light or in the dark at 20 °C for 20 d. Columns marked with the same letters were not significantly different based on LSD test at $P = 0.05$ for plants exposed to the dark; an asterisk indicates the difference between light and dark treatments.

Fig. 9. Cytokinin content of whole plants of creeping bentgrass grown under light or in the dark at 20 °C for 20 d. Columns marked with the same letters were not significantly different based on LSD test at $P = 0.05$ for plants exposed to the dark; an asterisk indicates the difference between light and dark treatments.

Fig. 10. Leaf chlorophyll content of *HSP18-ipt* whole plants of creeping bentgrass exposed to heat stress (35 °C) for 7 d. Columns marked with the same letters were not significantly different based on LSD test at $P = 0.05$ for plants exposed to heat stress; an asterisk indicates the difference between 20 and 35 °C.

Fig. 11. Leaf isopentenyladenine (iP/iPA) content of *HSP18-ipt* whole plants of creeping bentgrass exposed to heat stress (35 °C) for 7 d. Columns marked with the same letters were not significantly different based on LSD test at $P = 0.05$ for plants exposed to heat stress; an asterisk indicates the difference between 20 and 35 °C.

periods of stress. Hu et al. (2005) reported that tall fescue stayed green longer during the cooler temperatures of fall when transformed with a maize *ubiquitin-ipt* gene. Delayed leaf senescence is a desirable trait in perennial grass species used as turf or forage because maintaining green leaves provides an aesthetic function for turf and biomass production for forage grasses. Additional studies are needed to determine if the inhibition of leaf senescence in grass plants with *ipt* affects resource allocation to seeds, roots, and crowns.

We also created creeping bentgrass with the *ipt* gene ligated to a *HSP* promoter. To our knowledge, this is the first report of *HSP18-ipt* transformation in a perennial grass species, despite the importance of heat stress tolerance in cool-season grass species. Schmülling et al. (1989) were the first to show that with tobacco calluses, increased CK levels could be induced by heat treatment after transformation with *ipt* under the control of a *drosophila* (*Drosophila melanogaster*) *HSP* promoter. Smart et al. (1991) demonstrated that an *HSP* promoter from soybean (*Glycine max*) ligated to *ipt* could result in increased *ipt* expression after heat shock at 42 °C for 2 h and that this treatment led to higher levels of CK. Harding and Smigocki (1994) were the first to observe that transgenic tobacco with an *HSP-ipt* construct produced 2- to 4-fold higher levels of mRNA for small heat shock polypeptides and a wound-inducible glycine-rich protein, which may enhance heat tolerance.

Heat stress injury in plants results from a temperature-induced decline in carbohydrate accumulation, leaf senescence, and free radical damage, leading to a loss of membrane integrity. One of the earliest biochemical consequences of heat stress injury is a decline in tissue CK content (Liu and Huang, 2005). Veerasamy et al. (2007) pretreated creeping bentgrass with 10 μ M ZR before heat stress and found that leaf chlorophyll content, photochemical efficiency (F_v/F_m), and soluble protein content declined more slowly and protected plants from heat stress injury. The application of seaweed-based CK alleviated heat-induced decline in turfgrass quality, photochemical efficiency, and root viability in creeping bentgrass (Zhang and Ervin, 2008). Our results demonstrated that leaves of *HSP18-ipt* plants exposed to heat stress treatment (35 °C or higher) that had higher iPA content also exhibited lower electrolyte leakage and chlorophyll content compared with controls. Our *SAG12-ipt* plants also responded to heat stress, but not as strongly as the *HSP18-ipt* plants. *SAG12* is known to be

active only in older leaves and generally not directly responsive to stress-mediated signals (Weaver et al., 1998). The heat stress response that we measured occurred after 7 d at 35 °C, probably as a result of the senescence induced by this treatment.

In summary, transgenic creeping bentgrass with *SAG12-ipt* or *HSP18-ipt* maintained chlorophyll content and delayed the leaf senescence typically induced by dark or heat stress. *SAG12-ipt* and *HSP18-ipt* creeping bentgrass are potential new sources of grass germplasm with improved shade or heat tolerance. A field study is underway to examine performance of *SAG12-ipt* and *HSP18-ipt* lines under natural environmental conditions.

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