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Overexpression of phytochrome A and its hyperactive mutant improves shade tolerance and turf quality in creeping bentgrass and zoysiagrass

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Abstract Phytochrome A (phyA) in higher plants is known to function as a far-red/shade light-sensing photoreceptor in suppressing shade avoidance responses (SARs) to shade stress. In this paper, the *Avena PHYA* gene was introduced into creeping bentgrass (*Agrostis stolonifera* L.) and zoysiagrass (*Zoysia japonica* Steud.) to improve turf quality by suppressing the SARs. In addition to wild-type *PHYA*, a hyperactive mutant gene (*S599A-PHYA*), in which a phosphorylation site involved in light-signal attenuation was removed, was also transformed into the turfgrasses. Phenotypic traits of the transgenic plants were compared to assess the suppression of SARs under a simulated shade condition and outdoor field conditions after three growth seasons. Under the shade condition, the S599A-PhyA transgenic creeping bentgrass plants showed shade avoidance-suppressing phenotypes with a 45 % shorter leaf lengths, 24 % shorter internode lengths, and twofold increases in chlorophyll concentrations when compared with control plants. Transgenic zoysiagrass plants overexpressing *S599A-PHYA* also showed shade-tolerant phenotypes under the shade condition with reductions in leaf length (15 %), internode length (30 %), leaf length/width ratio (19 %) and leaf area (22 %), as well as increases in chlorophyll contents (19 %) and runner lengths (30 %) compared to control plants. The phenotypes of transgenic zoysiagrass were also investigated in dense field habitats, and the transgenic turfgrass exhibited shade-tolerant phenotypes similar to those observed under laboratory shade conditions. Therefore, the present study suggests that the hyperactive phyA is effective for the development of shade-tolerant plants, and that the shade tolerance nature is sustained under field conditions.

Keywords *Agrostis stolonifera* · Phytochromes · Shade avoidance · Transformation · Turfgrass · *Zoysia japonica*

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Abbreviations

phyA	Phytochrome A
phyB	Phytochrome B
SAR	Shade avoidance response
chl	Chlorophyll
NT	Non-transgenic plant
WL	White light
FR	Far-red light
RLC	Rapid light curve
ETR	Electron transport rate
VB	Vascular bundle
BSC	Bundle-sheath cells

Introduction

During photomorphogenesis, the growth and development of plants are profoundly affected by light of blue, red and far-red wavelengths (Neff et al. 2000; Chen et al. 2004; Han et al. 2007). The photomorphogenic effects of red and far-red light on plants are specifically mediated by the photoreceptor family of phytochromes: for example, phytochrome A (phyA) through E in *Arabidopsis thaliana* and phyA through C in *Oryza sativa* (Mathews 2010). Phytochrome exists in two distinct photochromic forms, red light-absorbing Pr and far-red light-absorbing Pfr (Quail et al. 1995; Rockwell et al. 2006; Nagatani 2010). The Pr form biosynthesized in plants absorbs red light, at which point the Pr form is converted to the Pfr form. The Pfr form is regarded as the physiologically active form by which light signals are transduced through interactions with a variety of upstream signal transducer proteins, ultimately regulating the downstream gene expression that mediate photomorphogenesis (Quail 2002; Wang and Deng 2003; Jiao et al. 2007; Bae and Choi 2008).

Shade avoidance response (SAR) in plants displaying etiolation and hypocotyl elongation is a prominent example of photomorphogenesis mediated by phytochromes, phyA for suppressing the SAR and phytochrome B (phyB) for promoting the SAR (Smith and Whitelam 1997; Neff et al. 2000; Devlin et al. 2003; Roig-Villanova et al. 2006; Franklin 2008). phyA and phyB are, respectively, necessary for recognition of shade as an environmental stress and survival of the plant in shade (Reed et al. 1994). Especially, phyA plays a photosensory role for a plant growing under conditions of shade stress (Johnson et al. 1994). Since the SAR causes significant crop-yield reductions, the *PHYA* gene has been introduced into crop plants such as tobacco, tomato, potato, wheat and rice to overcome the crop-yield losses (Boylan and Quail 1989; Heyer et al. 1995; Robson et al. 1996; Shlumukov et al. 2001; Sineshchekov et al. 2001; Kong et al. 2004; Garg et al. 2006). When constitutively expressed, phyA confers shade tolerance in plants, resulting in enhanced leaf expansion and growth with reduced elongation (for an example, see Robson et al. 1996). Most of PhyA transgenic plants displayed suppression of SAR with improved leaf expansion and growth, greening, and increased harvest indices for storage organs or seeds. These examples illustrate the importance of phyA for the suppression of shade avoidance reactions in crop plants (Sawers et al. 2005).

The light-signaling function of phyA in *A. thaliana* is apparently modulated by the Pfr-specific phosphorylation and dephosphorylation of a Ser residue in the hinge region of the photoreceptor molecule (Kim et al. 2004, 2005; Ryu et al. 2005; Phee et al. 2008). It is suggested that the phosphorylation might be involved in attenuating the light-signaling activity of phyA, whereas the Ser599Ala-phyA mutant functioned hyperactively by keeping the active Pfr pool in its unphosphorylated state. Furthermore, a phytochrome-specific protein phosphatase, PAPP5, positively regulates phytochrome interaction with downstream components and thus increases phytochrome signaling through the dephosphorylation of phytochrome (Ryu et al. 2005). Another phytochrome-specific protein phosphatase, PAPP2C that also positively regulates the light responses of plants, has been reported (Phee et al. 2008). Therefore, it is expected that transgenic plants overexpressing Ser599Ala-phyA could suppress the SAR more effectively through the action of the hyperactive phyA mutant, when compared with non-transgenic plant or transgenic plants with wild-type phyA.

In the present study, the *Ser599Ala-PHYA* mutant gene, as well as wild-type *PHYA* gene, was introduced into creeping bentgrass (*Agrostis stolonifera* L.) and Japanese lawn grass (*Zoysia japonica* Steud.) to suppress their SAR and to improve turf quality. Turfgrasses were chosen for the present study, because they grow in dense habitats, making extension growth due to SAR a serious problem, as it leads to a need for frequent mowing and consequently increases maintenance costs (Lee 1996; Chai and Stichlen 1998; Wang et al. 2001; Agharkar et al. 2007). In this study, transgenic plants with wild-type phyA or the hyperactive phyA mutant were successfully obtained and their phenotypic traits displayed by transgenic bentgrass and zoysiagrass cultivars were characterized under different light conditions. The present study shows that the overexpression of phyA in turfgrasses is effective for the suppression of SAR with the improvement of turf quality. Especially, the suppression of SAR shown in Ser599Ala-PhyA transgenic Arabidopsis plants was displayed in these two transgenic turfgrasses exhibiting superior turf qualities such as shorter and greener features with wider leaves, enhanced runner growth and tillering, accelerated sward growth, and high photosynthetic activities.

Materials and methods

Plant materials

Creeping bentgrass seeds (*A. stolonifera* L. cv. "Crenshaw") were purchased from KVBio Inc. (Seoul, Korea). The mature seeds of zoysiagrass (*Z. japonica* Steud.) were purchased from Mi Seong Turfgrass Co. (Chonnam, Korea) for producing transgenic plants. The seeds were stored at 4 °C prior to use.

Gene constructs used for transformation

The full-size cDNAs of wild-type (Wt) and Ser599Ala (S599A) *Avena sativa* (oat) *PHYA* were cloned into
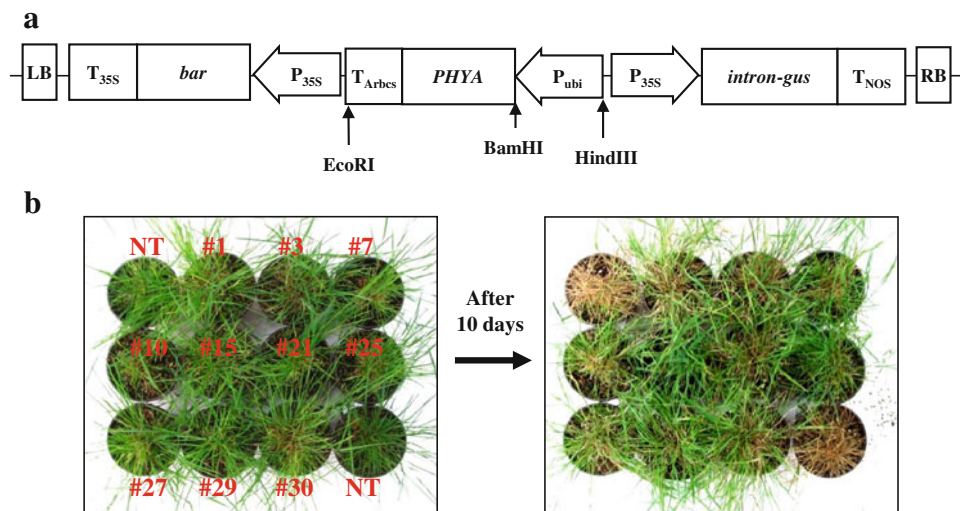
Fig. 1 Construct for transformation and herbicide resistance assay of putative transgenic plants. **a** T-DNA region of the binary vector plasmid pCAMBIA3301 harboring *PHYA*. RB, right border; LB, left border; P_{35S}, CaMV 35S promoter; P_{Ubi}, Ubiquitin promoter; *intron-gus*, GUS coding region containing a catalase intron insertion; *bar*, phosphinothricin acetyltransferase gene; *PHYA*, wild-type (*Wt*) or hyperactive mutant (*Ser599Ala* or *S599A*) *PHYA* gene; T_{35S}, CaMV 35S transcriptional terminator; T_{NOS}, *A. tumefaciens nos* gene terminator; T_{Arbcs}, *Arbcs* gene terminator. *Arrows* indicate directions of transcription. The *Eco*RI and *Hin*dIII restriction sites were used in Southern-blot analysis. **b** Herbicide resistance assay of representative transgenic plants. *Numbers in lanes* represent transgenic lines selected for this analysis (#1, #2, #3, #10 and #15 for Wt-PhyA transgenic plants, and (#21, #23, #25, #30 and #40 for S599A-PhyA transgenic plants). 0.8 % BASTA® was sprayed onto non-transgenic wild-type control plant (*NT*) and transgenic plants, and the herbicide resistance of the plants was determined 10 days later

pCAMBIA3301 from pFY122 (Boylan and Quail 1989) by digesting with *Bam*HI and *Eco*RI. The oat *PHYA* gene in pFY122 is composed of the amino-terminal half of "type 5" phytochrome (AP5; GenBank accession no. X03244) and the carboxy-terminal half of "type 3" phytochrome (AP3; GenBank accession no. X03242). pCAMBIA3301 carries the *bar* gene for herbicide resistance as a selectable marker, as well as an intron-containing *β*-glucuronidase gene (*intron-gus*) as a reporter; both genes are under control of the cauliflower mosaic virus (CaMV) 35S promoter, while *PHYA* genes are under the control of maize ubiquitin promoter (see Fig. 1a). The plasmid constructs were transformed into *Agrobacterium tumefaciens* EHA105 by the freeze–thaw method and then used for further turfgrass transformation. Throughout this paper, notations *PHYA* and phyA refer to gene and holoprotein of phyA, respectively, and transgenic plant overexpressing *Wt-PHYA* or *S599A-PHYA* refers to Wt-PhyA or S599A-PhyA.

Creeping bentgrass transformation

Tissue culture and genetic transformation of creeping bentgrass were performed as previously described (Kim et al. 2007; Han et al. 2009; Cho et al. 2011). During the transformation process, GUS staining assays were performed to check whether or not the embryogenic calli were transformed, as previously described (Kim et al. 2007). After transformation, plantlets with well-developed roots

were transferred to soil, grown under greenhouse conditions for 2 weeks, and then sprayed with 0.8 % (v/v) BASTA® (which contains 18 % ammonium glufosinate) to select for putative transgenic bentgrass plants. Herbicide resistance was determined 10 days later, and the herbicide-resistant plants were further analyzed.

Zoysiagrass transformation

The procedure for zoysiagrass transformation was performed according to the previous report (Toyama et al. 2003) with minor modifications. Mature seeds of *Z. japonica* Steud. were de-husked and surface-sterilized with 2 % sodium hypochlorite (NaOCl) containing 0.1 % (v/v) Tween 20 for 15 min. MS basal medium supplemented with 4 mg/L thiamine-HCl, 100 mg/L α-ketoglutaric acid, 2 mg/L 2,4-dichlorophenoxyacetic acid, 0.4 mg/L 6-benzyladenine, 30 g/L sucrose and 2 g/L gelrite (Duchefa Biochemie B.V.) was used for callus induction from mature seeds. Seeds were placed on the filter paper-impregnated media, and incubated at 28 °C in the dark to induce calluses. Induced calli were transferred to MS medium supplemented with 1 mg/L 2.4-dichlorophenoxy-acetic acid, 0.4 mg/L kinetin, 30 g/L sucrose and 2 g/L gelrite, and yellow, compact and friable embryogenic calli were visually selected for zoysiagrass transformation. *A. tumefaciens* cells were harvested and resuspended in 10 mL liquid infection media (calcium-free MS basal

medium supplemented with 0.1 mg/L BA, 10 g/L glucose, 30 g/L sucrose 100 mg/L acetosyringone, 0.02 % pluronic F68, pH 5.2). Embryogenic calli were then immersed in the bacterial suspension for 5 min. Following a quick drying on the filter paper, they were co-cultivated on a co-culture medium (calcium-free MS basal medium supplemented with 0.1 mg/L BA, 10 g/L glucose, 30 g/L sucrose, 100 mg/L acetosyringone, 2 g/L gelrite) at 28 °C in the dark. After 7 days of co-cultivation, the calli were washed with sterile water containing 1,000 mg/L carbenicillin, and transferred to the MS basal medium supplemented with 1 mg/L BA, 30 g/L maltose, 2 g/L gelrite, 250 mg/L carbenicillin and 2 mg/L bialaphos for shoot induction and pre-selection. The shoots were cultured for 4–8 weeks under 18 h light/6 h dark photoperiods of light intensity 30 μmol m^{-2} s^{-1}, and they were transferred to the MS medium supplemented with 30 g/L sucrose and 5 mg/L bialaphos for root induction and selection. Resulting well-developed plantlets were transferred to plastic pots filled with peat moss and perlite (1:1, w/w) and kept in the greenhouse. As described in the creeping bentgrass procedures, putative transgenic zoysiagrass plants were selected by herbicide resistance assays, and the herbicide-resistant plants were further analyzed.

Molecular analysis of transgenic turfgrasses

For Southern-blot analysis, creeping bentgrass and zoysiagrass genomic DNA were isolated from the leaves of greenhouse-grown plants and digested with either *Eco*RI or *Hin*dIII. DNA (20 μg/lane) was separated by using 0.8 % agarose gel electrophoresis and transferred to Amersham Hybond XL membranes (GE Healthcare). For probes, the *bar* gene was isolated from pCAMBIA3301 by restriction digestion with *Xho*I and then labeled with [α^{32}P] dCTP using the Radiprime^{TM} II Random Prime Labeling System (GE Healthcare). Unincorporated nucleotides were removed by using Microspin^{TM} G-50 columns (GE Healthcare). Southern hybridizations were performed by using Quick Hyb$^®$ (Stratagene). Pre-hybridization, hybridization and washing steps were carried out at 65 °C. After hybridization, the membranes were washed twice (5 min each) in washing solution I (2× SSC, 0.1 % SDS), followed by 5 min washing with solution II (1× SSC, 0.1 % SDS) and buffer III (0.5× SSC, 0.1 % SDS). The final wash was performed in 0.1× SSC and 0.1 % SDS for 15 min with agitation and then membranes were exposed to X-ray film (Agfa) at −70 °C for 2–3 days.

For RNA gel blot analysis, total RNA was extracted from plant leaves using TRIzol$^®$ reagent, according to the manufacturer's instructions (Invitrogen). Total RNA (10 μg) was separated on 1.2 % denaturing agarose gels in the presence of formaldehyde (1.8 mL of 37 % formaldehyde was added to 100 mL gel). Blotting, hybridization and washing of positively charged nylon membranes (Amersham Hybond XL; GE Healthcare) were conducted according to the manufacturer's instructions. RNA gel hybridizations were carried out with [α^{32}P] dCTP-labeled *bar* probes.

For Western-blot analysis, several leaves were taken and incubated between soaked Whatman papers for 24 h in the dark, followed by grinding the leaf tissues with Tissue-Ruptor (Qiagen) in liquid nitrogen, and 50 μg of protein samples were loaded onto 10 % SDS-PAGE gels for electrophoresis. The protein bands on the SDS-PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; GE Healthcare), and the membrane was incubated for 2 h with oat phyA-specific monoclonal antibody, oat22 (Cordonnier et al. 1983) or β-tubulin specific monoclonal antibody (Sigma), and developed using an ECL^{TM} Western blotting analysis system (GE Healthcare).

Phenotypic analysis of transgenic turfgrass plants

To investigate shade tolerance in plants, transgenic creeping bentgrass and zoysiagrass plants were grown in long day (LD, 16 h light/8 h dark) cycle at similar stages, fully grown leaves were then trimmed to the same lengths, and the plants were transferred to white light (WL, 100 μmol m^{-2} s^{-1}) or WL with supplementary far-red (WL + FR, 100 and 20 μmol m^{-2} s^{-1}, respectively) in a growth chamber. Cool white fluorescent light was used for WL and far-red LED light was used for FR (see FR-LED spectrum in Fig. S1). The plants were cultured for 20 days under the specified light conditions. The largest leaf from each individual plant was chosen to determine leaf and internode lengths.

For the investigation of phenotypes under field conditions, transgenic zoysiagrass plants from the laboratory were transferred to the greenhouse for acclimatization. All these lines were vegetatively planted during late spring and propagated to maturity during late August in different fields, including greenhouse (1 × 2 m lots, hereafter "greenhouse lot"), the field outside the lab (1 × 2 m lots, hereafter "lab lot"), and the government-approved GMO test field (2 × 2 m natural habitat plots located in Wimiri, Jeju, South Korea; hereafter "Wimiri field"). Since Wt-PhyA and S599A-PhyA transgenic lines were generated in different seasons, they were re-planted in the greenhouse lot and the standardized stock cultures showing healthy growth with proper acclimatization were maintained in the lab lot and Wimiri field for testing phenotypic evaluation. More than 2-year-old zoysiagrass plants after three growth seasons starting from April 2008 to December 2010 were used for the present study. The growth and

morphology of transgenic zoysiagrass plants were compared with those of the wild-type plants grown for four sessions in the Wimiri field, according to the previous report (Bae et al. 2008). Plant height was measured from the point where it was in contact with the ground to the apex by slightly stretching it. Internode length was determined from the crown part to the node of the third leaf of the first stoloniferous plant. Leaf blade width was measured on the widest part from the third leaf of the stoloniferous plant. The area of leaf blade was traced on graph sheets as previously described (Obiefuna and Ndubizu 1979). The ratio of leaf length divided by leaf width (leaf length/width) was calculated as an index of leaf size and shape as previously described (England and Attiwill 2006). In addition, the sward growth of zoysiagrass plants in the Wimiri field was measured after 2 weeks of mowing. Swards are considered as small leaf blades remaining after mowing treatment, and the sward growth responses to cutting treatments are considered as one of the important growth parameters for turfgrass phenotypic characterization. The total heights of the swards were measured for all transgenic zoysiagrass lines after the mowing.

To assess the variations in leaf blade width and chlorophyll contents at the cellular level, the cell arrangement of transgenic and control plants were compared by transverse sections. For this anatomical study, the leaf blades were randomly sampled, and cross-sections across the widest part of the leaf blades were made manually and fixed, and stored in formalin–acetic acid–alcohol to observe the variations in number of vascular bundles (VBs) and bundle sheath cells (BSCs), and mesophyll tissue arrangement (Carmo-Silva et al. 2009). For SEM observations, the cross-sections were air-dried, coated with platinum, and photographed. In addition, runner lengths and tiller numbers were calculated from zoysiagrass plants. The plants were uprooted and runner lengths were measured. The tiller numbers were calculated per square foot by counting the number of tillers produced per inflorescence axis.

Measurements of chlorophyll contents

Chlorophyll (chl) content of creeping bentgrass was measured as reported (Kim et al. 2004). Chlorophyll a and b contents were calculated from the equations ($13.36 \times \lambda_{664} - 5.19 \times \lambda_{648}$) for chlorophyll a, and ($27.43 \times \lambda_{648} - 8.12 \times \lambda_{664}$) for chlorophyll b, respectively. In the case of zoysiagrass plants, the leaves were extracted with buffered 80 % aqueous acetone (pH 7.8) and chlorophyll content was estimated as described previously (Porra et al. 1989). In addition, relative chlorophyll contents of zoysiagrass plants in LED chamber, greenhouse lot and Wimiri field were measured with a portable chlorophyll meter

(SPAD-502Plus; Konica Minolta) in terms of SPAD (soil plant analysis development) values.

Chlorophyll fluorescence analysis

Chlorophyll fluorescence was assessed using an IMAGING-PAM Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany) as previously described (Schreiber et al. 1994). The IMAGING-PAM was operated in conjunction with a computer and WinControl software (Walz). Using the leaf tissues of 0.5 mm size in diameter (in order to create rapid light response curves for photosynthesis), stepwise increases in the intensity of actinic light were applied in a series of 40 s pulses. The quantum yield of the photochemical energy conversion in photosystem II (PS II) can be estimated using the empirical fluorescence parameters $(F_m' - F)/F_m' = \Delta F/F_m'$, and the relative electron transport rate (ETR) was calculated from $\Delta F/F_m' \times$ PPFD $\times c$, in which PPFD is the photosynthetic photon flux density of the incident active radiation and the constant c (absorption factor for the measurement) is considered to be 1.0 (Genty et al. 1989). A saturating light pulse was applied to the samples at the end of each illumination period. All experiments were conducted at room temperature.

Statistical analysis

Means and standard errors were used throughout the study and the values were assessed using a parametric moods median test ($n = 3$) (Snedecor and Cochran 1989). The data were analyzed for variance by Duncan's multiple range test (DMRT) using the SPSS statistical programme version 17.0 in necessary places.

Results

Production of transgenic creeping bentgrass overexpressing *PHYA*

Embryogenic callus tissue derived from mature seeds has been used for *Agrobacterium*-mediated transformation of several monocotyledonous species and is considered to be the best target tissue for transformation (Cheng et al. 2004). In the present study, *Agrobacterium*-mediated transformation was performed with embryogenic calli derived from the mature seeds of creeping bentgrass (*A. stolonifera* L. cv. "Crenshaw"), using an established method for creeping bentgrass regeneration and transformation (Kim et al. 2007; Han et al. 2009). With this method, transgenic creeping bentgrass plants with wild-type *Avena PHYA* (*Wt-PHYA*) or a hyperactive mutant *PHYA* (*S599A-PHYA*) gene
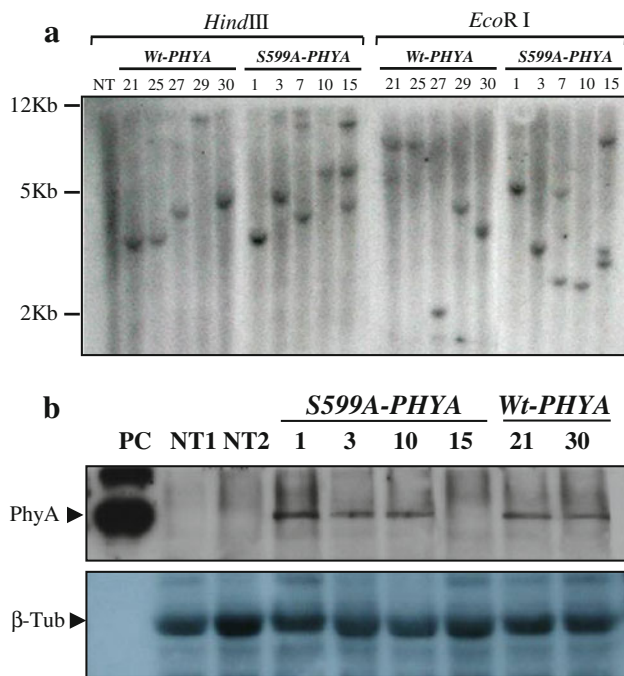
a

b

Fig. 2 Molecular analysis of transgenic creeping bentgrass plants with wild-type *PHYA* (*Wt-PHYA*) and a hyperactive mutant *PHYA* (*S599A-PHYA*). **a** Southern-blot analysis of representative transgenic plants of *Wt-PHYA* and *S599A-PHYA*. Genomic DNA from transgenic creeping bentgrass plants was digested with either *Hind*III or *Eco*RI, and the *bar* gene was used as a probe. *NT* non-transgenic plant. *Numbers in lanes* represent transgenic lines selected for the analyses. **b** Western-blot analysis of representative transgenic plants. Crude extracts of 24-h dark-treated leaves were isolated and loaded onto 10 % SDS-PAGE for the analysis. Oat phyA-specific monoclonal antibody, oat22, or β-tubulin specific monoclonal antibody was used for the detection of phyA and β-tubulin (β-*Tub*), respectively. *NT1* and *NT2* non-transgenic plants, *PC* positive control (i.e. purified oat phyA)

were successfully generated. Since the binary vector used for the transformation contained the herbicide resistance *bar* gene along with *PHYA* (Fig. 1a), putative transgenic creeping bentgrass plants were detected by herbicide resistance assays in the greenhouse (Fig. 1b). 13.6 and 8.0 % efficiencies for transforming creeping bentgrass were achieved with *Wt-PHYA* and *S599A-PHYA*, respectively, which are close to the success rates previously reported (Han et al. 2009; Cho et al. 2011). These putative herbicide-resistant plants all possessed both *bar* and *PHYA*, as demonstrated by genomic PCR analysis (Fig. S2). Southern-blot analysis confirmed the integration of the transgene, and Western-blot analysis with oat phyA-specific monoclonal antibody oat22 showed the expression of phyA proteins in the transformed plant lines (Fig. 2). Since protein expression levels are closely related to the function of phyA, the transgenic lines with a single transgene integration showing similar protein expression levels were selected for further analysis, for example, lines #21 and

#30 of the Wt-PhyA transgenic plants and lines #3 and #10 of the S599A-PhyA transgenic plants. These lines were used for further phenotypic analysis.

Enhanced shade tolerance of transgenic bentgrass overexpressing *Wt-PHYA* or *S599A-PHYA*

The representative Wt-PhyA and S599A-PhyA transgenic lines displaying comparable phyA expression levels were used for the study of their phenotypic traits under WL (16/8 h photoperiod at 100 μmol m^{-2} s^{-1}) or a simulated shade light (WL + FR, WL with supplementary far-red light, WL:FR = 100:20 μmol m^{-2} s^{-1}) in a growth chamber. By inserting a FR-LED illumination system in the middle of the growth chamber, the upper part is illuminated by fluorescent WL and the lower part is maintained as a shaded (WL + FR) condition. Thus, it is possible to compare the phenotypes of each transgenic line under light and shade conditions in the same plant growth chamber.

Shade avoidance responses include extension growths such as elongated leaves, petioles and internodes, and retardation of chloroplast development and chlorophyll synthesis. Thus, the lengths of leaf and internode (i.e. the length between two leaves), and chlorophyll contents from each transgenic plant of Wt-phyA and S599A-phyA under both light and shade conditions were investigated. As control plants, a transgenic bentgrass line with pCAM-BIA3301 (i.e., "vector only" control, line #3 described in Kim et al. 2007) and non-transgenic plant (NT) were included in this study. Leaf and internode lengths and chlorophyll concentrations were measured during 20 days of growth (Fig. S3). In the light condition, non-transgenic and the control transgenic plant with pCAMBIA3301 showed similar growths of leaves and internodes, while Wt-PhyA transgenic plants displayed approximately 2.0 % shorter leaf length and about 4.3 % shorter internode length relative to the control plants (light in Fig. 3a, b; Table 1). Since strong dependency of phyA function on the photoreceptor amounts is known (Boylan and Quail 1991; Whitelam et al. 1993), these results suggest that the overexpression of phyA in transgenic plants increased the total activity of phyA compared with non-transgenic and control plants. More importantly, S599A-PhyA transgenic plants displayed more reductions in leaf and internode lengths (5.7 and 12.2 %) than Wt-PhyA transgenic plants, indicating that S599A-phyA is hyperactive in creeping bentgrass compared to wild-type phyA.

Under the simulated shade condition (i.e. WL + FR), Wt-PhyA and S599A-PhyA transgenic plants showed significantly suppressed leaf and internode extensions compared with non-transgenic and control plants, indicating the suppression of the SAR (shade in Fig. 3a, b; Table 1).
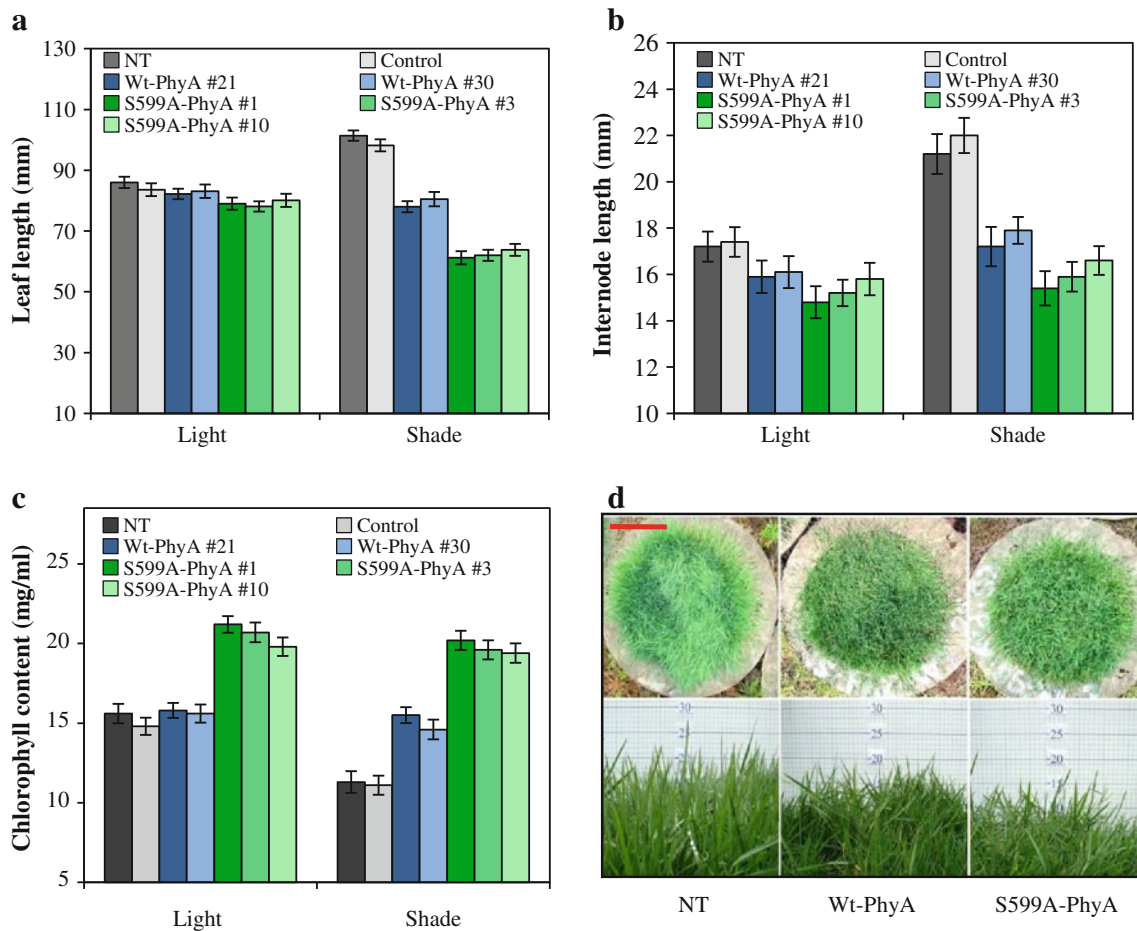
Fig. 3 Phenotypic analyses of transgenic creeping bentgrass plants under light or shade conditions. Leaf lengths (**a**), internodes lengths (**b**) and chlorophyll contents (**c**) were measured from plants grown for 20 days under white light or simulated shade condition. *Light* white light condition (WL, 100 μmol m^{-2} s^{-1}); *shade* white light with supplementary far-red light (FR) as a simulated shade condition (WL + FR, 100 + 20 μmol m^{-2} s^{-1}). *NT* non-transgenic bentgrass plant, *control* transgenic bentgrass plant with pCAMBIA3301 (i.e., "vector only" control), *Wt-PhyA* transgenic bentgrass plant with wild-type *PHYA* (lines #21 and #30), *S599A-PhyA* transgenic bentgrass plant with *S599A-PHYA* (lines #1, 3 and #10). Each value indicates the mean ± SE (*n* = 16 for **a** and **b**, *n* = 10 for **c**). **d** Phenotypes of creeping bentgrass plants grown in dense field conditions. *Bar* 20 cm

Table 1 Relative SAR suppression in transgenic turfgrasses (versus control) overexpressing *Wt-PHYA* or *S599A-PHYA* under light and shade light conditions

Transgenic plants	Decrease (%) in leaf lengths		Decrease (%) in internode lengths		Increase (%) in chlorophyll contents	
	Light	Shade	Light	Shade	Light	Shade
Creeping bentgrass Wt-PhyA	2.0 ± 0.03[d]	13.3 ± 0.13[c]	4.3 ± 0.090[d]	18.0 ± 0.20[d]	2.3 ± 0.16[c]	35.6 ± 1.2[b]
Creeping bentgrass S599A-PhyA	5.7 ± 0.19[b]	44.9 ± 0.20[a]	12.2 ± 0.31[c]	24.4 ± 0.27[c]	30.9 ± 0.30[a]	94.0 ± 6.1[a]
Zoysiagrass Wt-PhyA	3.5 ± 0.11[c]	4.2 ± 0.090[d]	16.5 ± 0.31[b]	28.0 ± 0.12[b]	3.2 ± 0.21[c]	13.9 ± 0.05[cd]
Zoysiagrass S599A-PhyA	7.5 ± 0.42[a]	16.0 ± 0.34[b]	24.5 ± 0.42[a]	35.1 ± 0.95[a]	10.0 ± 0.06[b]	19.1 ± 0.08[c]

Apparent comparison only (see text and experimental procedures). Each value indicates the mean ± SE (*n* = 30–40). Means within a column followed by the same superscript letters are not significant at *P* = 0.05 according to DMRT. Light, white light of 100 μmol m^{-2} s^{-1} with 16/8 h photoperiod; Shade, white light with far-red light (100 and 20 μmol m^{-2} s^{-1}). Decreased or increased values from 100 % were calculated by setting the amount of control plant as 100 %. Control plant was an herbicide-resistant transgenic bentgrass or zoysiagrass line with pCAMBIA3301 (i.e., "vector only" control). Wt-PhyA, transgenic plants with *Wt-PHYA*; S599A-PhyA, transgenic plants with *S599A-PHYA*

The SAR suppression was particularly strong in S599A-PhyA transgenic plants, which showed approximately 45 % reduction in leaf lengths and about 24 % reduction in internode lengths under shade condition compared with control plants, thus exhibiting their phenotypic features comparable to those grown under light condition.

Moreover, the S599A-PhyA transgenic plants were shorter in leaf lengths (~30 %) and internodes (~7 %) than the Wt-PhyA transgenic plants, showing a stronger suppression of the SAR compared to Wt-PhyA transgenic plants.

Shade avoidance responses also include retarded chloroplast development and reduced chlorophyll synthesis, so chlorophyll contents of plants were further investigated. Even under light condition, the S599A-PhyA plants showed approximately 31 % increase in chlorophyll content relative to control plants (light in Fig. 3c; Table 1). On the other hand, the chlorophyll level of the Wt-PhyA transgenic plant was only slightly increased (~2 %) compared with control plants. Under the shade condition, the chlorophyll contents of the non-transgenic and control plants declined due to the SAR, whereas Wt-PhyA and S599A-PhyA transgenic plants produced comparable levels of chlorophylls to those grown under light condition (shade in Fig. 3c; Table 1). The S599A-PhyA plants showed approximately twofold increase in chlorophyll contents relative to control plants under the shade condition, suggesting again that S599A-phyA is efficient for the suppression of the SAR. Furthermore, the shade-tolerant phenotypes (i.e. shorter leaf lengths and internodes) of S599A-PhyA transgenic plants were also exhibited similarly under natural light conditions in a dense-habitat field (Fig. 3d). Therefore, these results could provide the basis for the development of commercially useful shade-tolerant plants using the hyperactive phyA mutant gene, S599A-PHYA.

Production of transgenic zoysiagrass overexpressing PHYA

In addition to transgenic cool-season creeping bentgrass, transgenic warm-season zoysiagrass (Z. japonica Steud.) were also generated by introducing pCAMBIA3301 harboring Wt-PHYA or S599A-PHYA using the Agrobacterium-mediated transformation procedure reported previously (Toyama et al. 2003). After transformation, putative transgenic zoysiagrass lines were screened by herbicide resistance assays (data not shown). Southern-blot analysis confirmed transgene integration in the transgenic lines (Fig. 4a), and RNA gel blot analysis confirmed similar mRNA expression levels of the transgene bar in the transgenic lines (Fig. 4b). Most transgenic plants had single transgene integration with similar expression levels of bar. However, the protein expression levels of phyA somewhat varied among the transgenic lines (Fig. 4c). Thus, Wt-PhyA #1 and S599A-PhyA #14 and #18 lines displaying similar phyA protein expression levels with single transgene integration were selected for further studies. Especially, because of the gene flow problem of transgenic creeping bentgrass (Mallory-Smith and Zapiola 2008; Zapiola et al. 2008), only the transgenic zoysiagrass lines were used for field tests.
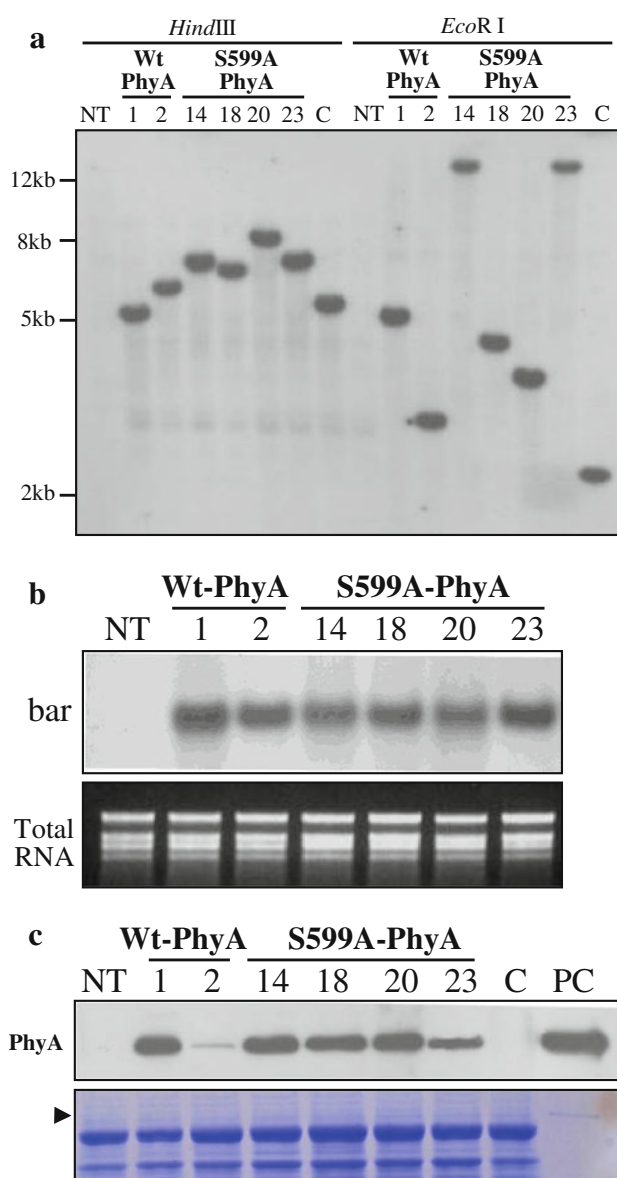

Fig. 4 Molecular analysis of transgenic zoysiagrass plants with Wt-PHYA and S599A-PHYA. **a** Southern-blot analysis of representative transgenic plants of Wt-PHYA and S599A-PHYA. NT non-transgenic plant, C herbicide-resistant control plant as a positive control (i.e. transgenic zoysiagrass plants with pCAMBIA3301). **b** RNA gel blot analysis of representative transgenic plants. The bar gene was used as a probe and total RNA gel was shown as a loading control. **c** Western-blot analysis of representative transgenic plants. SDS-PAGE was shown as a loading control. NT non-transgenic plant, PC positive control (i.e. purified oat phyA). Arrowhead indicates the position of phyA band

Enhanced shade tolerance of transgenic zoysiagrass overexpressing S599A-PHYA

To investigate the shade tolerance of transgenic zoysiagrass plants, plant heights, internodes, leaf lengths and widths, and chlorophyll contents in transgenic zoysiagrass

lines grown in greenhouse and Wimiri field were measured under natural daylight conditions. Since no significant differences in the morphology and growth parameters were shown between non-transgenic plant and transgenic herbicide-resistant control plant with pCAMBIA3301 (Toyama et al. 2003; Bae et al. 2008), the phenotypes of Wt-PhyA or S599A-PhyA transgenic plants were compared relative to the latter. Both transgenic zoysiagrass plants with Wt-phyA or S599A-phyA showed similar shade-tolerant phenotypes to transgenic bentgrass plants, which included decreases in SAR-induced leaf and internode elongation and suppression of SAR-induced decrease of chlorophyll biosynthesis (Table 1). Furthermore, transgenic zoysiagrass plant with Wt-phyA exhibited shorter plant heights and internode lengths, and greener leaves in both dense-habitat greenhouse lot and Wimiri field, compared with control plant (Fig. 5; Table S1). Notably, the S599A-PhyA transgenic lines displayed approximately 30–35 % shorter plant height, ∼25 % reduced internode length, and ∼15 % increased chlorophyll level compared with control plant, displaying features indicative of the suppression of the SAR. In particular, S599A-PhyA transgenic plant showed stronger shade-tolerant phenotypes than Wt-PhyA transgenic plant. These results further suggest that S599A-phyA elicited stronger shade-tolerant phenotypes in zoysiagrass plants than Wt-phyA.

As described earlier with creeping bentgrass plants, it was investigated whether S599A-phyA could suppress the SAR in transgenic zoysiagrass plants under a simulated shade condition (i.e. WL + FR) by using 738-nm LED light in a growth chamber. The S599A-PhyA transgenic zoysiagrass lines showed 35 % shorter internode length and 16 % shortened plant height under the simulated shade condition (Fig. 6a, b; Table 2). Consistently, the chlorophyll concentrations were higher in both Wt-PhyA (14 %) and S599A-PhyA (19 %) transgenic plants than in control plant (Fig. 6c). In addition, the S599A-PhyA transgenic plants showed decreases in leaf length/width ratio (by 18 %) and leaf area (by 22 %) under simulated shade condition, when compared with the control plant (Table 2).

To ascertain the possible functional role of the increased chlorophyll concentrations in the PhyA transgenic lines, rapid light curves (RLCs) as expressed by the relative ETR were examined and the correlation between the high chlorophyll contents and the photosynthetic activity in S599A-PhyA transgenic plants were investigated. The RLCs provide insight into the photosynthetic light saturation properties of the leaf sample (Schreiber et al. 1994). When recorded with light-adapted leaf tissues, the RLC data reflect the dependency of the relative photosynthetic ETR on quantum flux density under given light conditions. Results showed the electron transport capacity with saturation at
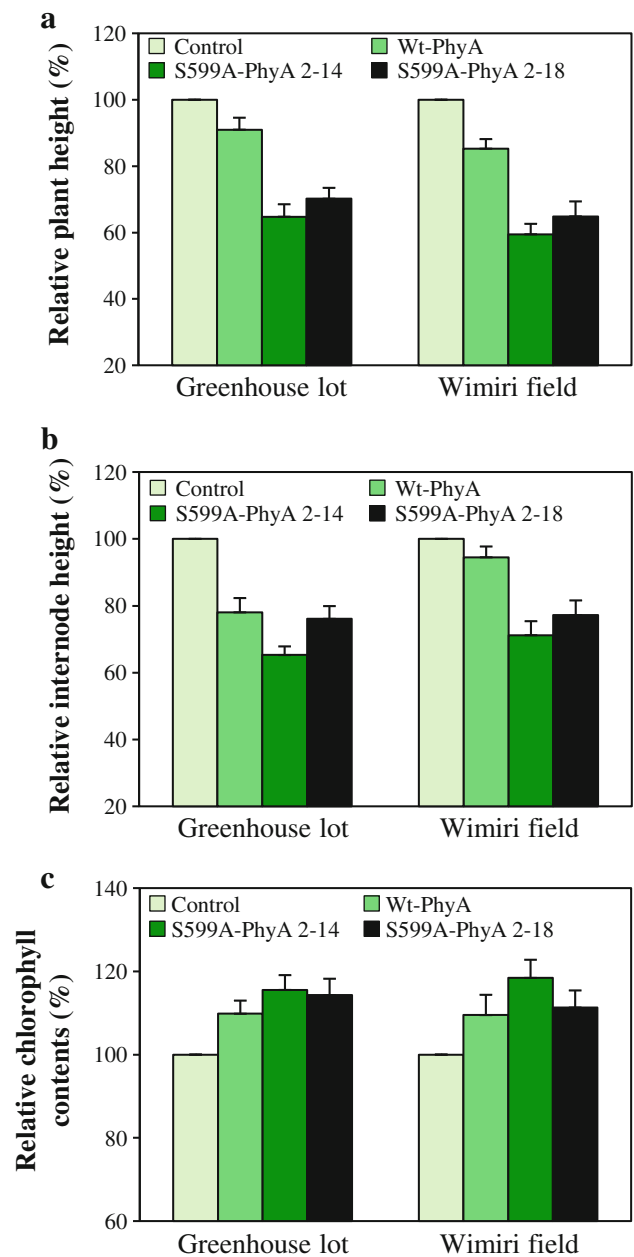

Fig. 5 Variations in plant heights, internode lengths and relative chlorophyll contents of zoysiagrass plants under dense-habitat greenhouse lot and Wimiri field. **a** Relative plant heights were calculated by setting the height of control plant as 100 % (control heights; 26.3 cm in greenhouse lot and 25.8 cm in Wimiri field). **b** Relative internode lengths were calculated by setting the internode of control plant as 100 % (control internode lengths; 5.4 mm in greenhouse lot and 6.1 mm in Wimiri field). **c** Relative chlorophyll contents were calculated by setting the amount of control plant as 100 % (control total chlorophyll contents; 826.5 nmol g^{-1} FW in greenhouse lot and 886.5 nmol g^{-1} FW in Wimiri field). Each value indicates the mean ± SE (n = 15–19). *Control* herbicide-resistant transgenic zoysiagrass with pCAMBIA3301 (i.e., "vector only" control), *Wt-PhyA* transgenic zoysiagrass plant with *Wt-PHYA* (line #1), *S599A-PhyA* transgenic zoysiagrass plants with *S599A-PHYA* (lines #2–14 and #2–18)
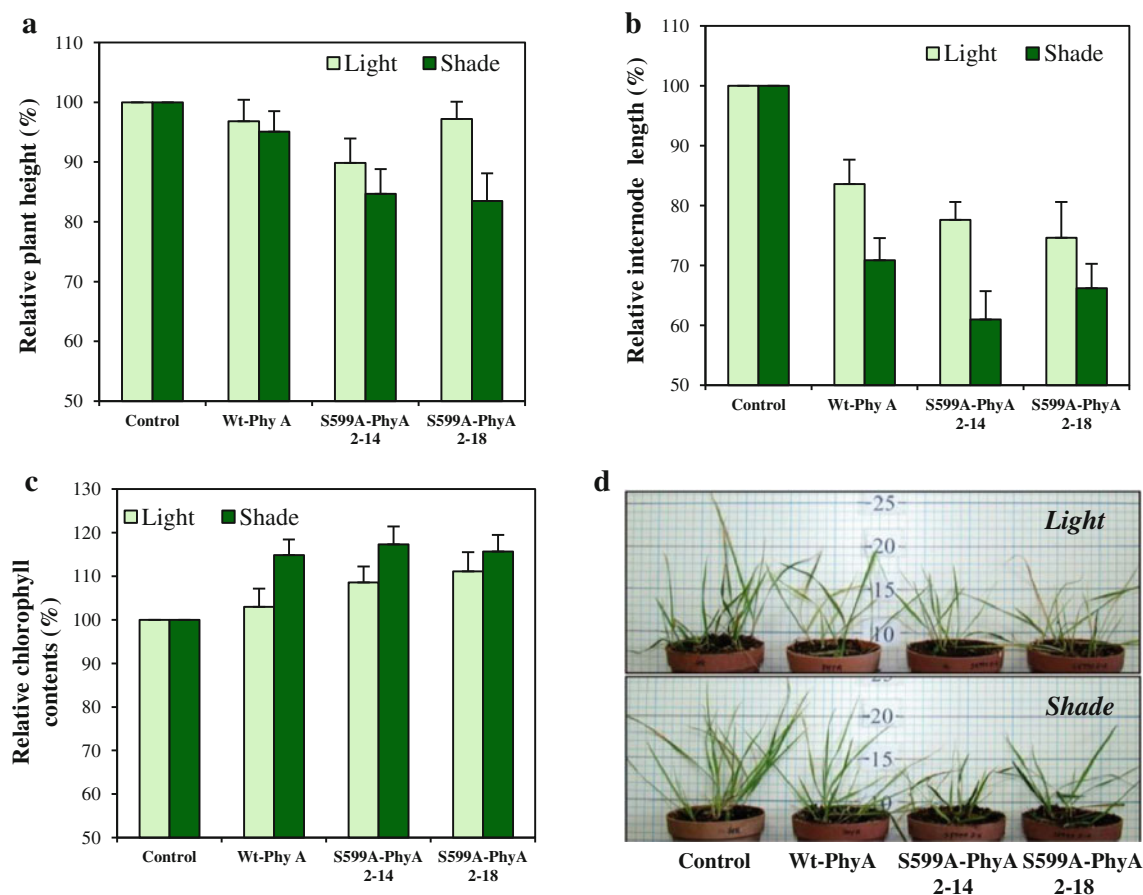
Fig. 6 Variations in plant heights, internode lengths and chlorophyll contents of transgenic zoysiagrass lines under light or shade conditions. **a** Relative plant heights were calculated by setting the height of control plant as 100 % (control plant heights; 19.0 cm in the light condition and 26.5 cm in the shade condition). **b** Relative internode lengths were calculated by setting the internode of control plant as 100 % (control internode lengths; 6.7 mm in the light condition and 1.03 mm in the shade condition). **c** Relative chlorophyll contents were calculated by setting the amount of control plant as 100 % (control total chlorophyll contents; 26.5 SPAD units in the light condition and 26.2 SPAD units in the shade condition). **d** Phenotypes of zoysiagrass plants on pots after 100 days of growth under light or simulated shade conditions. *Control* herbicide-resistant transgenic zoysiagrass with pCAMBIA3301, *Wt-PhyA* transgenic zoysiagrass plant with *Wt-PHYA*, *S599A-PhyA 2–14 and 2–18* transgenic zoysiagrass plants with *S599A-PHYA*. Each value indicates the percentage ± SE ($n = 10$–12)

around 300 μmol m^{-2} s^{-1} under both WL and shade (WL + FR) conditions (Figs. 7, S4). Under the WL condition, maximum photosynthetic capacity (ETR$_{max}$) of S599A-PhyA transgenic plant was higher than Wt-PhyA transgenic plant (Fig. S4). The ETRs under light condition decreased at the light intensities of ≥500 μmol m^{-2} s^{-1}. In the simulated shade condition, the rise of the curve with S599A-PhyA transgenic plant is also proportional to the capacity of light absorption, and the ETR values of S599A-PhyA transgenic plants were significantly higher than those of control plant at the light intensities of ≥400 μmol m^{-2} s^{-1} (Fig. 7). These results appear to correlate the higher chlorophyll contents in transgenic zoysiagrass lines with the mutant phyA. Therefore, these results suggest that the shade-tolerant phenotype induced by S599A-phyA (i.e. reduced plant heights and internodes) was

not due to reduction or perturbation in general metabolic efficiency and photosynthetic capacity, but rather reflected a specific effect of S599A-phyA function.

Leaf anatomical studies were also performed to verify the biological mechanism behind the wider leaf of S599A-PhyA transgenic lines at SEM level, which showed 65 % of the leaf width occupied by VBs and BSCs in zoysiagrass (Fig. S5). All the examined S599A-PhyA transgenic zoysiagrass lines exhibited the VBs surrounded by the greater number of BSCs, compared to the Wt-PhyA transgenic and control plants (Fig. 8; Table 3). Maximum of 32 VBs was recorded in S599A-PhyA transgenic lines compared to 28 in Wt-PhyA transgenic line. Furthermore, three different types of VBs, namely midrib (1st order VB), large sized (2nd order VB) and small sized VBs (3rd Order VB) were also observed (Fig. S5). In all the three types of VBs, a

Table 2 Comparison of leaf morphological variables among transgenic zoysiagrass lines under simulated shade (LED) conditions

Lines	Leaf width (mm)	Leaf length (cm)	Leaf length/width ratio	Leaf area (cm²/leaf)
Control				
Light	4.58 ± 0.18[e] (100)	17.2 ± 0.66[c] (100)	3.64 ± 0.45[c] (100)	49.8 ± 2.35[cd] (100)
Shade	4.58 ± 0.14[e] (100)	18.1 ± 1.37[a] (100)	3.87 ± 0.34[b] (100)	65.7 ± 1.26[a] (100)
Wt-PhyA				
Light	4.63 ± 0.09[d] (101.4)	16.7 ± 1.14[d] (97.8)	3.51 ± 0.34[d] (96.6)	43.5 ± 2.47[f] (87.1)
Shade	4.79 ± 0.15[a] (103.9)	18.2 ± 0.96[a] (100.2)	3.86 ± 0.17[b] (98.63)	59.9 ± 3.22[b] (91.4)
S599A-PhyA 2–14				
Light	4.71 ± 0.12[b] (102.3)	15.2 ± 0.84[e] (87.4)	3.14 ± 0.19[ef] (87.1)	41.5 ± 2.08[g] (83.2)
Shade	4.8 ± 0.15[a] (104.9)	16.4 ± 1.01[d] (91.1)	3.35 ± 0.2[e] (86.3)	49.1 ± 1.97[cd] (73.9)
S599A-PhyA 2–18				
Light	4.67 ± 0.14[cd] (102.3)	15.6 ± 0.75[e] (89.6)	3.24 ± 0.21[e] (89.6)	45.9 ± 2.71[e] (92.0)
Shade	4.64 ± 0.18[d] (103.4)	17.7 ± 0.67[b] (96.4)	3.72 ± 0.15[bc] (95.7)	48.2 ± 2.1[d] (73.4)

Each value indicates the mean ± SE ($n = 15$–19). Means within a column followed by the same superscript letters are not significant at $P = 0.05$ according to DMRT. Light, white light of 100 μmol m^{-2} s^{-1} with 16/8 h photoperiod; Shade, white light with far-red light (100 and 20 μmol m^{-2} s^{-1}). Values within parenthesis are percentage of variation and measured using the value of control plant as 100 %. Control, herbicide-resistant transgenic zoysiagrass with pCAMBIA3301; Wt-PhyA, transgenic zoysiagrass plants with *Wt-PHYA*; S599A-PhyA 2–14 and 2–18, transgenic zoysiagrass plants with *S599A-PHYA*
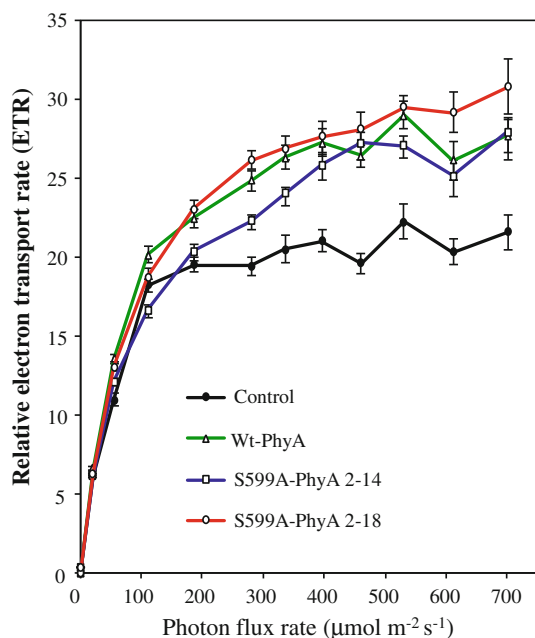

Fig. 7 Rapid light responses of zoysiagrass plants under a simulated shade condition. Chlorophyll fluorescence quenching was detected by a saturating pulse method from the leaves of plants grown under a simulated shade condition (WL + FR, 150 and 12 μmol m^{-2} s^{-1}, respectively). The values are expressed as the mean ± SE ($n = 10$). *Control* herbicide-resistant transgenic zoysiagrass plant with pCAM-BIA3301, *Wt-PhyA* transgenic zoysiagrass plant with *Wt-PHYA*, *S599A-PhyA 2–14 and 2–18* transgenic zoysiagrass plants with *S599A-PHYA*

greater number of BSCs in the S599A-PhyA transgenic lines were recorded (Table 3). The relative abundance of VBs and BSCs thus appear to functionally correlate the

SAR suppression with the higher chlorophyll contents of S599A-PhyA zoysiagrass leaves.

Improved turf quality of transgenic zoysiagrass overexpressing *S599A-PHYA*

To evaluate the turf quality of transgenic zoysiagrass, the variations in the runner lengths and tiller numbers of zoysiagrass plants grown in the lab lot condition were investigated. The runners cover the ground surface vigorously by producing tillers, and are important for shoot initiation and vegetative propagation in determining the sward growth quality of turfgrasses. In all the zoysiagrass lines studied, the formation of new runners was recorded after approximately 75 days of growth maintenance under both light and shade conditions. After 100 days, longer runners were formed in the S599A-PhyA transgenic plants than the other plants. The runners of S599A-PhyA 2–14 zoysiagrass plants reached 11.2 cm in length, which is much longer than control and Wt-PhyA transgenic plants (Fig. S6a). The density of tillers was also measured with plants grown under Wimiri field condition by counting matured inflorescence axis per area. The S599A-PhyA transgenic lines showed a higher number of inflorescence axis per square foot than other lines (Fig. S6b). Lastly, the variations in sward growth responses of transgenic zoysiagrass to mowing treatments were measured after 3 and 17 days of mowing under the lab lot condition (Figs. 9, S7). The S599A-PhyA transgenic plants showed uniform growth of new swards after moving compared with control plants. Interestingly, the results showed 30 and 24 % decreases in the sward heights of S599A-PhyA and

Fig. 8 Scanning electron microscopic (SEM) view of third order vascular bundles of zoysiagrass plants grown under greenhouse lot conditions. *Control* herbicide-resistant transgenic zoysiagrass with pCAMBIA3301, *Wt-PhyA* transgenic zoysiagrass plant with *Wt-PHYA*, *S599A-PhyA 2–14 and 2–18* transgenic zoysiagrass plants with *S599A-PHYA*, *BSC* photosynthetic bundle sheath cells, *BUL* bulliform cells
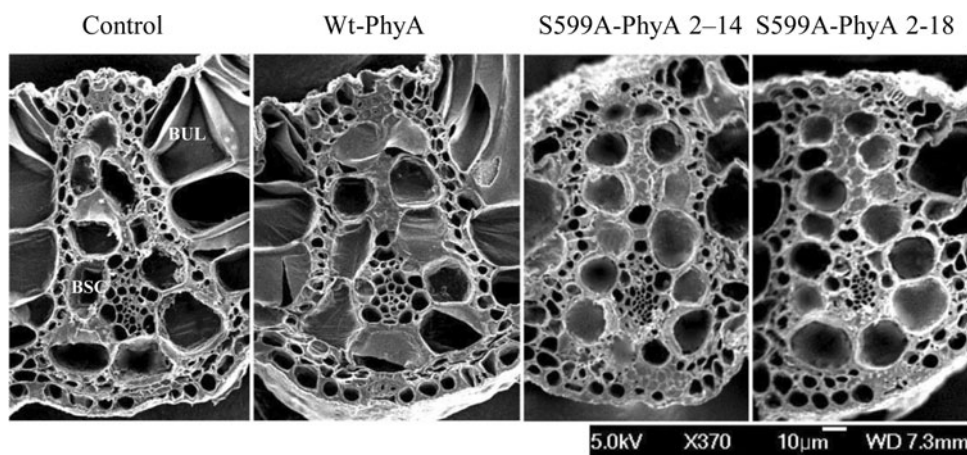

Table 3 Variations in the number of vascular bundles (VBs) and bundle sheath cells (BSCs) in cross-sections (CSs) of zoysiagrass plants grown in lab lot

Lines	VBs/CS of leaf sample	BSCs per first order VB	BSCs per second order VB	BSCs per third order VB
Control	26.5 ± 0.42^d	15.5 ± 0.44^{bc}	8.5 ± 0.22^d	12.9 ± 0.37^b
Wt-PhyA	28.2 ± 0.74^c	15.9 ± 0.26^b	8.9 ± 0.34^c	13.0 ± 0.30^b
S599A-PhyA 2–14	32.5 ± 0.48^a	16.8 ± 0.75^a	11.8 ± 0.25^a	16.8 ± 0.41^a
S599A-PhyA 2–18	31.7 ± 0.72^b	16.7 ± 0.15^a	10.7 ± 0.27^b	16.9 ± 0.35^a

Measurements were made from cross-sections in the widest part of leaf blade. Means within a column followed by the same superscript letters are not significant at $P = 0.05$ according to DMRT. Control, herbicide-resistant transgenic zoysiagrass with pCAMBIA3301; Wt-PhyA, transgenic zoysiagrass plant with *Wt-PHYA*; S599A-PhyA 2–14 and 18, transgenic zoysiagrass plants with *S599A-PHYA*. Values are mean ± SE of ten leaf samples in three replicates

Wt-PhyA transgenic lines compared to the control plant, respectively (Fig. 9). The same growth trend of sward heights and leaf lengths were recorded for S599A-PhyA and Wt-PhyA lines even after 2 months. Interestingly, during the early winter season, a delay in the browning (i.e. senescence) of S599A-PhyA transgenic leaves were observed under the greenhouse lot condition (Fig. S8). An average delay of 25 days in the senescence of the S599A-PhyA transgenic zoysiagrass plants was observed in response to natural cold stress. Overall, the S599A-PhyA transgenic lines displayed short and uniform sward growth responses, higher tiller numbers in the form of inflorescences, and delayed senescence compared with other zoysiagrass plants.

Discussion

Turfgrasses are commonly classified into two major groups, cool-season and warm-season turfgrass. The cool-season turfgrasses are well adapted to temperatures between 16 and 25 °C and are widely distributed throughout the cool humid or sub-humid regions. Creeping bentgrass (*A. stolonifera* L.) is an economically important cool-season turfgrass whose fine texture, dense growth and tolerance of very low cutting heights have led to its extensive use on putting

greens and fairways on temperate climate golf courses (Warnke 2003). A representative warm-season turfgrass is zoysiagrass (*Z. japonica* Steud.), which is the most important species of turfgrass in East Asia, including Korea, Japan, China, and in other temperate zones, due to its extraordinary advantages for lawns and playing fields as well as its resistance to drought (Toyama et al. 2003; Ge et al. 2006). Recently, genetic transformation methods have been adopted for efficient introduction of useful traits from a broader range of sources and within an economically viable time frame (Lee 1996; Chai and Stichlen 1998; Wang et al. 2001; Wang and Ge 2006). Meanwhile, several approaches are on the way to produce environmentally friendly genetically modified plants. In the present study, *Wt-PHYA* or *S599A-PHYA* along with *bar* were successfully introduced into both creeping bentgrass (Figs. 1, 2) and zoysiagrass (Fig. 4) for enhanced shade tolerance and turf quality, as well as effective control of weeds using herbicide resistance.

The functional importance of phytochrome phosphorylation in plant light signaling has been demonstrated (Kim et al. 2002, 2004; Ryu et al. 2005; Phee et al. 2008; Han et al. 2010). Serine-599 of oat phyA is phosphorylated in a Pfr-preferential manner and the Ser599Ala mutant of oat phyA is physiologically hyperactive in transgenic

Fig. 9 Phenotypic variations in the sward heights of transgenic zoysiagrass. **a** The sward heights of transgenic zoysiagrass after 3 and 17 days of mowing under the lab lot conditions. **b** Phenotypes of transgenic zoysiagrass after 17 days of mowing. *Bar* 10 cm. *Control* herbicide-resistant transgenic zoysiagrass with pCAMBIA3301, *Wt-PhyA* transgenic zoysiagrass plant with *Wt-PHYA*, *S599A-PhyA 2–14 and 2–18* transgenic zoysiagrass plants with *S599A-PHYA*. Each value indicates the mean ± SE ($n = 4$–6)
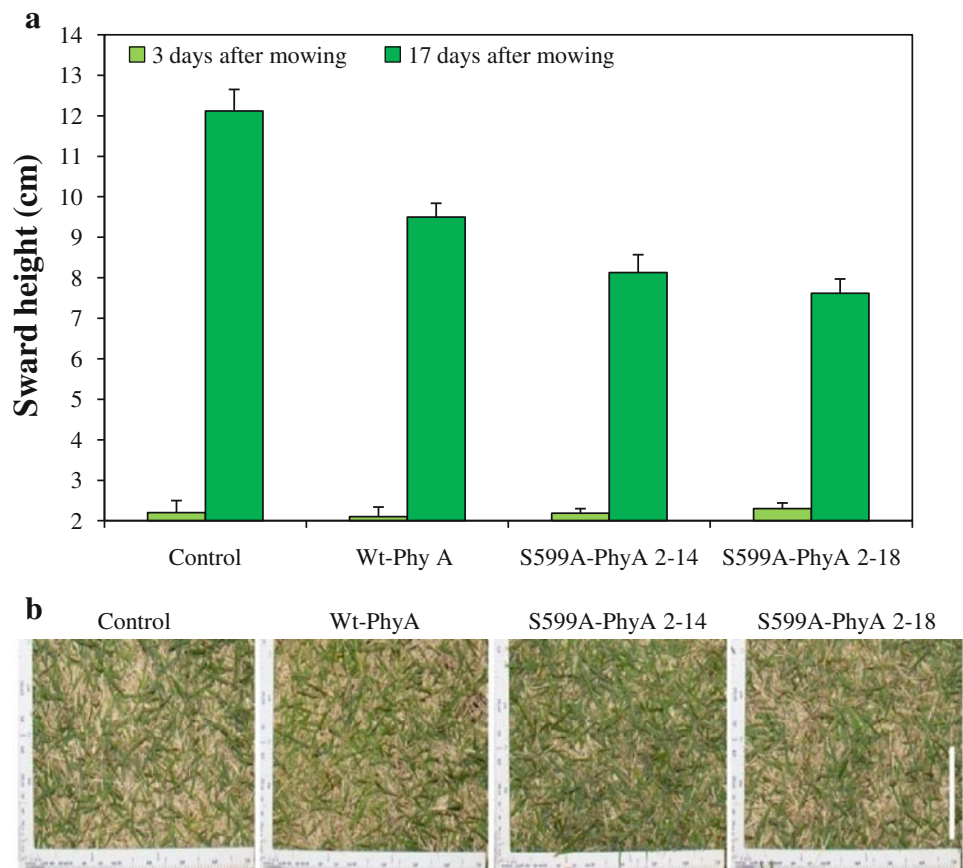
A. thaliana, as the Ser599Ala mutation keeps phyA in its active Pfr pool (Kim et al. 2004, 2005). The phytochrome mutant S599A-phyA conferred strong shade tolerance to *Arabidopsis* plants. In fact, transgenic plants with the mutant phyA was much more shade-tolerant than that with wild-type phyA in *Arabidopsis* (Kim et al. 2004). In the present study, the *S599A-PHYA* as well as *Wt-PHYA* was introduced into creeping bentgrass and zoysiagrass for phenotypic function studies and to develop turfgrass cultivars with commercial potential and environmental benefits.

The transformation of creeping bentgrass needs less time than that of zoysiagrass, with transformation efficiency substantially higher for the former than for the latter. However, a higher percentage of single transgene integration was observed with zoysiagrass transformation than with creeping bentgrass transformation (Figs. 2a, 4a). This might be due to the genome size of creeping bentgrass, which is significantly larger than that of zoysiagrass (Bonos et al. 2002). The expression levels of phyA proteins were also higher in zoysiagrass than in creeping bentgrass plants (Figs. 2b, 4c), and more variation of phyA protein levels were observed in zoysiagrass than in bentgrass. Therefore, transgenic lines showing similar phyA protein levels with single copy integration were selected for further phenotypic analysis.

Shade avoidance responses in plants include extension growth of stems and petioles at the expense of leaf growth, retarded chloroplast development with reduced chlorophyll levels, accelerated flowering, and reduced storage organ deposition (Smith and Whitelam 1997; Franklin 2008). Both transgenic Wt-PhyA and S599A-PhyA creeping bentgrass plants showed reduced leaf and internode lengths but increased chlorophyll levels under shade conditions (Fig. 3), consistent with shade-tolerant phenotypes. Furthermore, the chlorophyll levels of S599A-PhyA transgenic bentgrass remained at similar levels to those of light-grown plants even after 20 days of shade treatment, while those of non-transgenic and control plants were significantly reduced (Fig. 3c). These results suggest that S599A-phyA suppresses the SAR efficiently in creeping bentgrass, even compared to wild-type phyA.

The transgenic zoysiagrass plants with *S599A-PHYA* also showed typical shade-tolerant phenotypes under both greenhouse and Wimiri field conditions, with shorter heights and greener leaves than the control zoysiagrass plant (Fig. 5). Promisingly, shade tolerance under simulated shade or dense-growth conditions appears to be stronger in both Wt-PhyA and S599A-PhyA transgenic plants than the control plant. In particular, the shade tolerance of S599A-PhyA transgenic zoysiagrass was more

pronounced in the Wimiri field than in the greenhouse lot, suggesting that S599A-phyA functions efficiently in suppressing the SAR in the dense-growth conditions. Furthermore, S599A-PhyA transgenic zoysiagrass contained higher chlorophyll levels in dense-growth conditions than control and Wt-PhyA transgenic plants (Fig. 5c). Like plants grown under Wimiri field and greenhouse lot conditions, S599A-PhyA transgenic zoysiagrass also showed suppression of the SAR under the simulated shade condition with FR-LED (Fig. 6; Table 2). S599A-PhyA transgenic zoysiagrass also showed a delay of senescence during the early winter session (Fig. S8). The delayed senescence might be a manifestation of the cold tolerance of the S599A-PhyA transgenic zoysiagrass, a warm-season grass, as phytochrome plays an important role in cold tolerance in *Arabidopsis* (Franklin and Whitelam 2007).

The ETR values of S599A-PhyA transgenic plants were significantly higher (25–35 %) than those of non-transgenic or control plants, suggesting that the shortened phenotypes of leaves and internodes induced by S599A-phyA were not from reduced metabolic efficiency but from the effects of S599A-phyA expression (Fig. 7). These results support the hypothesis that the phosphorylation and dephosphorylation of oat phyA protein at Ser598 plays a prominent role in suppressing the SAR in both creeping bentgrass and zoysiagrass plants. The effective photosynthesis and high chlorophyll contents of these plants might also be helpful in the reduction of carbon dioxide in the atmosphere as environmental friendly trait (Long et al. 2004). In addition, the S599A-PhyA leaves contain relatively high numbers of VBs, surrounded by a greater number of BSCs, which results in the increased chlorophyll levels and greener leaves (Fig. 8). This is significant, because C4 plants perform photosynthesis through the cooperation between mesophyll and bundle sheath chloroplast cells (Furumoto et al. 1999). SEM data showed no significant differences in the mesophyll tissue organization between S599A-PhyA transgenic zoysiagrass and control plants, but a significantly higher number of VBs and BSCs were found in the S599A-PhyA transgenic plants (Table 3). This variation in the structure of vascular tissues may be due to the overexpression of S599A-phyA or Wt-phyA, contributing to the overall shade tolerance traits of the PhyA transgenic plants.

Runner lengths in the S599A-PhyA transgenic lines were relatively long under both WL and simulated shade conditions (Fig. S6a). In the Wimiri field, the S599A-PhyA zoysiagrass plants also showed 20 % more tillers per square foot than controls (Fig. S6b). In response to mowing, the S599A-PhyA and Wt-PhyA zoysiagrass exhibited better sward growth quality than the control plant (Figs. 9, S7). During sward growth, these cultivars displayed better ground coverage than the control plant (Fig. 9b). This is consistent with the variation of leaf area index (LAI)

controlled by the sward characteristics such as the number of leaves per tiller and that of tillers per area (Sbrissia et al. 2010). The long runners and greater number of tillers in the S599A-PhyA zoysiagrass transgenic plants are favorable sward growth traits for speedy vegetative propagation and ground coverage as well as for improved seed harvest.

The S599A-phyA mutant seems to suppress the SAR more effectively than the Wt-phyA mutant (Tables 1, 2), consistent with the hyperactive nature of S599A-phyA demonstrated in *A. thaliana*. Certain shade tolerance traits such as chlorophyll levels conferred by the mutant phyA appear to be more prominent in creeping bentgrass than in zoysiagrass under simulated shade conditions (Table 1). However, in terms of plant height, internode length, leaf length and width, overexpression of S599A-PhyA showed stronger suppression of SAR in transgenic zoysiagrass than in creeping bentgrass. Collectively, these results indicate economically significant trait advantages for both S599A-phyA transgenic turfgrass species. In particular, the warm session turfgrass, zoysiagrass, showed commercially useful additional traits such as increased photosynthetic activity, enhanced cold tolerance and delayed senescence in the winter.

In conclusion, the present study demonstrates that S599A-phyA confers commercially useful traits to turfgrasses. Since herbicide-resistant turfgrass with the *bar* gene showed several advantages (Toyama et al. 2003; Kim et al. 2007), the pyramiding of *bar* and the hyperactive *PHYA* in bentgrass and zoysiagrass cultivars reported here could provide economical and environmental advantages over wild-type turfgrasses by increasing photosynthesis and reducing the frequency of mowing, herbicide application required, and improving the sward quality of turfgrasses.

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