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Communication

Antisense expression of a staygreen gene (*SGR*) delays leaf senescence in creeping bentgrass

Ok-Jin Hwang[1], Yun-Jeong Han[1], Nam-Chon Paek[2], and Jeong-Il Kim[1,*]

[1]Department of Biotechnology and Kumho Life Science Laboratory, Chonnam National University, Gwangju 500-757, Korea
[2]Department of Plant Science, Plant Genomics and Breeding Institute, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 151–921, Korea

ABSTRACT: Loss of chlorophyll is the visible symptom of leaf senescence and staygreen refers to the delayed leaf senescence in plants. The staygreen gene (*SGR*) in rice (*Oryza sativa* L.) has been identified as its mutation maintains greenness during leaf senescence, and encodes a chloroplast protein required for the initiation of chlorophyll breakdown in plants. In this study, we isolated a rice *SGR*-homologous gene in creeping bentgrass (*Agrostis stolonifera* L.), and transgenic creeping bentgrass plants were obtained by introducing pCAMBIA3301 vector harboring antisense *SGR* gene under control of the senescence-specific SAG12 promoter. Transgenic plants were selected by herbicide resistance assays and genomic integration of the transgenes was confirmed by PCR analysis. Subsequent analyses demonstrated the staygreen phenotype of the transgenic creeping bentgrass plants with decreased chlorophyll loss during leaf senescence. These results suggest that the antisense *SGR* expression in creeping bentgrass delays leaf senescence, which provides a way to develop genetically engineered turfgrass varieties with the commercially useful staygreen trait.

Staygreen refers to the heritable delayed foliar senescence character in plants, usually due to impaired or delayed chlorophyll catabolism.[1] During leaf senescence, chlorophyll (Chl) is converted to colorless breakdown products in a multi-step catabolic pathway, resulting in loss of green color in leaves.[2-4] Staygreen genes encode members of chloroplast-located proteins that are likely to function in dismantling of photosynthetic chlorophyll–protein complexes. These activity is considered as a prerequisite for chlorophyll degradation during senescence.[5]

Staygreen mutants are delayed in leaf senescence and have been identified from different plant species, including rice (*Oryza sativa* L.). For an example, an amino acid substitution from valine residue to methionine in rice *SGR* (Os09g36200) has shown the *staygreen* (*sgr*) mutant phenotype.[6] On the other hand, overexpression of *SGR* in transgenic *Arabidopsis* has led to enhanced chlorophyll breakdown and precocious senescence.[7] Subsequently, mutations in the *SGR* gene in other plant species including pepper (*Capsicum annuum* L.), pea (*Pisum sativum* L.) and tomato (*Solanum lycopersicum* L.) have shown to cause a delayed loss of Chl in plants undergoing both natural and dark-induced senescence.[8] These studies indicate that *SGR* expression is induced at the onset of leaf senescence, concomitant with chlorophyll breakdown. Therefore, suppression of *SGR* expression during leaf senescence might confer the staygreen phenotype to plants.

The objective of this study was to develop transgenic creeping bentgrass plants with the staygreen phenotype by antisense expression of the *SGR* gene. Creeping bentgrass (*Agrostis stolonifera* L.) is an economically important cool-season turfgrass, with a fine texture, dense growth and tolerance to low cutting heights that have made it suitable for extensive use on putting greens and fairways of golf courses in temperate climates.[9] As the utilization area of the turfgrass species increases recently, the staygreen trait is very attractive to be manipulated by genetic transformation. In this study, we isolated the fragment of the *SGR* gene in creeping bentgrass, and produced transgenic creeping bentgrass plants with staygreen phenotype successfully by introducing pCAMBIA3300 vector harboring antisense *SGR* gene under control of a senescence-specific promoter.

The fragment of the *SGR* gene in creeping bentgrass was isolated by PCR with a pair of degenerate primers, 5′-ACKTACACDCTNACDCACAGYGA-3′ (forward) and 5′-TTGGARTGGAARTARACCCAMAC-3′ (reverse) (K, G/T; D, G/A/T; N, All, Y, C/T; R, G/A; M, C/A). The degenerate primers were designed on the basis of conserved nucleotide sequences of *SGR* genes in plants, and cDNA prepared from senescent leaves of creeping bentgrass was used as a template. The 329 bp fragment of *SGR* in creeping bentgrass showed highly conserved sequence (90.3 % identity) to the rice *SGR* gene (**Fig. 1**). With this *SGR* fragment of creeping bentgrass, a gene cassette consisting of antisense *SGR* under the control of *SAG12* promoter[10] and *ARBCS* gene terminator[11] (i.e., *SAG12::anti-SGR*) was subcloned into the

***To whom correspondence should be addressed.**
E-mail: kimji@chonnam.ac.kr

binary vector pCAMBIA3300 using *Hin*dIII and *Eco*RI (**Fig. 2A**). *SAG12* is one of senescence-associated genes (*SAGs*) and used a marker gene for senescence. The *SAG12* promoter has been applied for senescence-induced gene expression in plants.[12] The plasmid construct was transformed into *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw method,[13] followed by creeping bentgrass transformation.

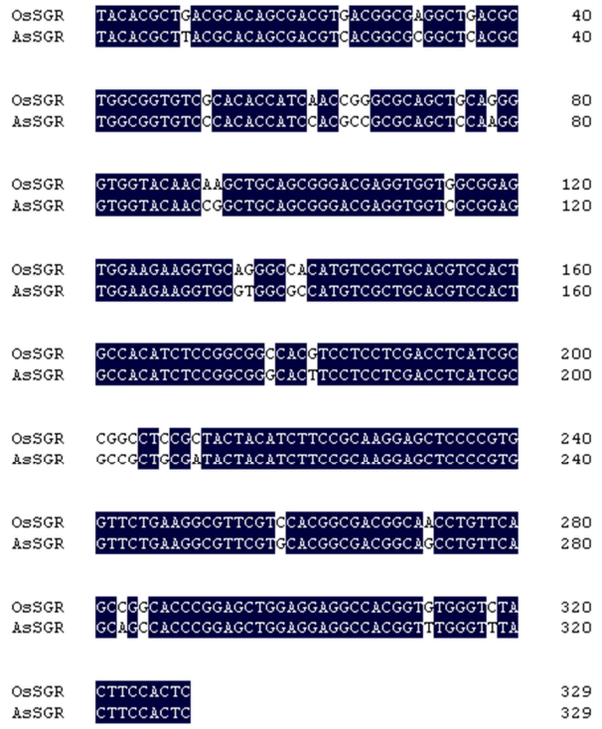

Figure 1. Alignment of the *SGR* nucleotide sequences between rice and creeping bentgrass. OsSGR, *SGR* sequence from *Oryza sativa* L.; AsSGR, *SGR* sequence from *Agrostis stolonifera* L. Nucleotide sequence of 329 bp *AsSGR* that was used for antisense construct was aligned with that of *OsSGR*, and identical sequences were shown in boxes.

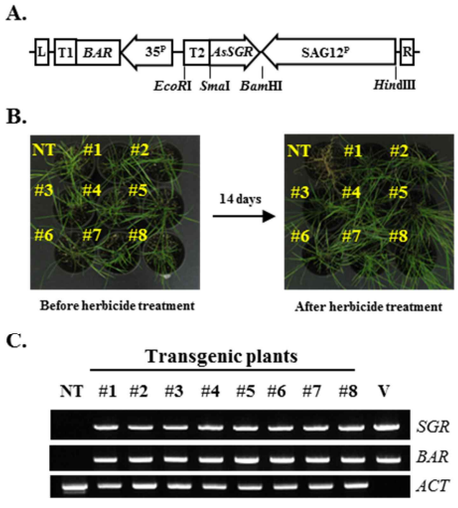

Figure 2. Production of transgenic creeping bentgrass plants with antisense expression of the *AsSGR* gene. (A) T-DNA region of the binary vector plasmid pCAMBIA3300 harboring a gene cassette consisting of antisense *SGR* under the control of *SAG12* promoter (i.e., *SAG12::anti-SGR*). L, left border; R, right border; $35S^P$, CaMV 35S promoter; $SAG12^P$, senescence-specific *SAG12* promoter; *AsSGR*, 329 bp fragment of *SGR* from creeping bentgrass (*Agrostis stolonifera* L.) in reverse orientation; *BAR*, phosphinotricin acetyltransferase gene coding region; T1, CaMV 35S gene terminator; T2, *ARBCS* gene terminator. Arrows in promoters indicate directions of transcription. (B) Herbicide resistance assay. Numbers in lanes represent putative transgenic plants selected for this analysis. 0.4 % BASTA® was sprayed onto non-transformed wild-type control plant (NT) and the transgenic plants, and the herbicide resistance of the plants was determined 14 days later. (C) Genomic PCR analysis. The coding regions of the *SGR* and *BAR* transgenes were amplified by PCR from genomic DNA. The actin gene (*ACT*) was shown as a loading control of the genomic DNA. NT, non-transformed control plant; V, pCAMBIA3300 harboring *SAG12::anti-SGR* that was used for transformation.

Seeds of the 'Crenshaw' cultivar of creeping bentgrass were used for genetic transformation, as previously described.[14-16] Since the binary vector used for the transformation contained the *BAR* herbicide resistance gene, putative transgenic creeping bentgrass plants were identified by BASTA® resistance assay. After transformation, plantlets with well-developed roots were transferred to soil and grown for 2 weeks under greenhouse conditions prior to herbicide resistance assays. Following herbicide treatment with .4% (v/v) BASTA® (which contains 18% glufosinate ammonium), non-transformed wild-type control plant (NT) died within 2 weeks, whereas all of the putative transformants were resistant to the herbicide (**Fig. 2B**). These results indicate that the *BAR* gene was expressed in these transgenic plants.

To verify the insertion of *SGR* and *BAR* transgenes, PCR assays on genomic DNA were performed and integration of both sequences was confirmed in all herbicide-resistant plants (**Fig. 2C**). No amplified band was observed in the non-transformed (NT) control plant. For this genomic PCR analyses, genomic DNA was isolated from the leaves of greenhouse-grown plants, and the coding regions for the *BAR* and *SGR* transgenes were amplified from either genomic DNA or a positive control vector, using the following sets of primers: 5′-GCACGAATGGTTCTCTTGTGAATAAAC-3′ (forward) and 5′-TCCAAGGGTGGTACAACC-3′ (reverse) for *SGR* and 5′-CTACCATGAGCCCAGAACGACG-3′ (forward) and 5′-CTGCCAGAAACCCACGTCATGCCAGTTC-3′ (reverse) for *BAR*. The actin (*ACT*) gene of creeping bentgrass was also amplified using the same template and the primers 5′-AACTGGGACGACATGGAGAAGATA-3′ (forward) and 5′-CGTCAGGGAGCTCGTAGTTCTTC-3′ (reverse), and then run as a loading control of genomic DNA. The results of

genomic PCR analyses confirmed that the creeping bentgrass plants obtained by the *Agrobacterium*-mediated genetic transformation are all transgenic plants.

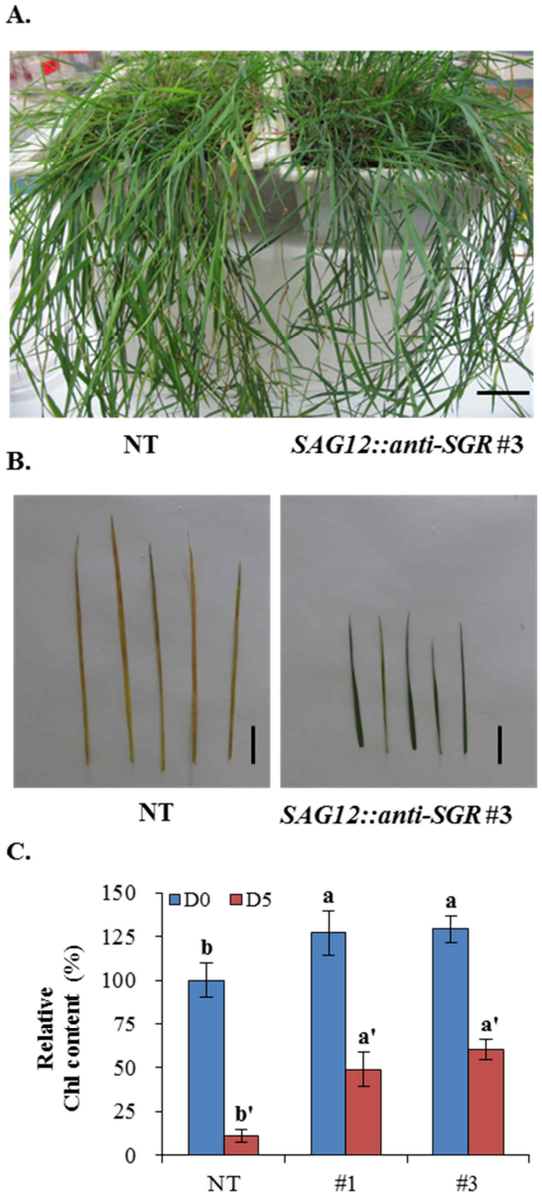

Figure 3. Phenotypic and senescence analyses of transgenic creeping bentgrass plants with *SAG12::anti-SGR*. (A) Apparent phenotype of fully-grown creeping bentgrass plants. NT, non-transformed control plant. Bar, 5 cm. (B) Dark-induced senescence assays. Leaves were detached from 4-week-old soil-grown plants and incubated in dark at 25°C for 5 days. (C) Measurement of chlorophyll content. Leaves before (D0) and 5 days after dark incubation (D5) were extracted with 80% acetone and absorbance was measured at 645 nm (A_{645}) and 663 nm (A_{663}). Total chlorophyll content was calculated from the equation: total chlorophyll (μg/mL) = $20.2 \times A_{645} + 8.02 \times A_{663}$. Relative chlorophyll content was calculated by setting the chlorophyll content of non-transformed control plant (NT) to 100 %. Error bars indicate standard errors ($n = 3$), and means with different letters are significantly different at $P < 0.05$, using Duncan.

After having the transgenic plants, we first investigated apparent phenotypes in greenhouse. Generally, all of the transgenic bentgrass plants appeared normal under greenhouse conditions and were morphologically indistinguishable from wild-type plants. However, fully-grown transgenic plants were greener than the non-transformed control plant (**Fig. 3A**). Approximately 27-29% increases in chlorophyll content were observed in the transgenic plants. In addition, transgenic bentgrass plants had shorter leaf size than the control plant (**Fig. 3B**). However, differences in the plant growth rates were not observed between the control and transgenic plants (data not shown), which suggests that the short leaf phenotype might be due to the antisense expression of the *SGR* gene. At this point, it is not clear why the plants with the antisense *SGR* expression displayed shorter leaves than the control plant, so further studies will be necessary to elucidate the relationship between the short leaf phenotype and the antisense *SGR* expression. Next, to examine the staygreen phenotype, leaf longevity of the plants was investigated by dark-induced leaf senescence assays. For this, the third fully expanded leaves from each plant were placed on wet 3M paper and incubated in darkness at 25 °C for 5 days. After the treatment of dark-induced senescence, the leaves of control plant turned yellow, whereas those of transgenic plants remained green (**Fig. 3B**). Moreover, chlorophyll content of transgenic plants was approximately 4-5 fold higher than the control plant after 5 days of dark-induced leaf senescence treatment (**Fig. 3C**). When the chlorophyll loss was compared after 5 days of dark incubation, the transgenic plants lost about 50-60 % Chl content while the control plant lost approximately 90%. These results suggest that chlorophyll degradation is delayed by the antisense expression of *SGR*, which is consistent with the previous report in rice.[6] Therefore, the transgenic bentgrass plants with *SAG12::anti-SGR* displayed the staygreen phenotype with decreased chlorophyll loss during leaf senescence.

In conclusion, the present study demonstrates that the antisense expression of the *SGR* gene confers a staygreen phenotype to creeping bentgrass, which provides a method to develop genetically engineered turfgrass varieties with the commercially useful staygreen trait.

KEYWORDS: Creeping bentgrass, Leaf senescence, Staygreen.

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