



Molecular cloning and characterization of a chlorophyll degradation regulatory gene (*ZjSGR*) from *Zoysia japonica*

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ABSTRACT. The stay-green gene (*SGR*) is a key regulatory factor for chlorophyll degradation and senescence. However, to date, little is known about *SGR* in *Zoysia japonica*. In this study, *ZjSGR* was cloned, using rapid amplification of cDNA ends-polymerase chain reaction (PCR). The target sequence is 831 bp in length, corresponding to 276 amino acids. Protein BLAST results showed that *ZjSGR* belongs to the stay-green superfamily. A phylogenetic analysis implied that *ZjSGR* is most closely related to *ZmSGR1*. The subcellular localization of *ZjSGR* was investigated, using an *Agrobacterium*-mediated transient expression assay in *Nicotiana benthamiana*. Our results demonstrated that *ZjSGR* protein is localized in the chloroplasts. Quantitative real time PCR was carried out to investigate the expression characteristics of *ZjSGR*. The expression level of *ZjSGR* was found to be highest in leaves, and could be strongly induced by natural senescence, darkness, abscisic acid (ABA), and methyl jasmonate treatment. Moreover, an *in*

vivo function analysis indicated that transient overexpression of *ZjSGR* could accelerate chlorophyll degradation, up-regulate the expression of *SAG113*, and activate ABA biosynthesis. Taken together, these results provide evidence that *ZjSGR* could play an important regulatory role in leaf chlorophyll degradation and senescence in plants at the molecular level.

Key words: *Zoysia japonica*; *SGR*; Chlorophyll degradation; Leaf senescence

INTRODUCTION

Chlorophyll degradation is the most obvious phenomenon of plant senescence, and many of the underlying biochemical steps have been elucidated (Hörtensteiner, 2006). The identification of *stay-green* mutants in several plant species has made it possible to further investigate the underlying mechanism (Armstead et al., 2006; Park et al., 2007; Zhou et al., 2011). Stay-green (SGR) proteins play important roles in the regulation of plant chlorophyll degradation and senescence, and they have been characterized in *Arabidopsis thaliana* (Ren et al., 2007), *Medicago truncatula* (Zhou et al., 2011), and *Solanum lycopersicum* (Luo et al., 2013). Constitutively increased *SGR* levels resulted in accelerated chlorophyll degradation in *Arabidopsis* leaves (Ren et al., 2007). Transient overexpression of *OsSGR* by agro-infiltration in tobacco leaves induced subsequent senescence (Park et al., 2007). *MtSGR* was possibly involved in alfalfa nodule development and senescence (Zhou et al., 2011). However, the role of *SGRs* in regulating chlorophyll degradation and senescence is still largely unknown, particularly in non-model plants.

The identification of senescence-associated genes (*SAGs*) paved the way for elucidating leaf senescence at the transcriptional level (Schippers et al., 2015). *SAG113* was found to be up-regulated during the leaf senescence process (Zhang et al., 2012). Abscisic acid (ABA) has been verified to repress chloroplast biosynthesis genes and to induce genes that promote chlorophyll degradation during plant senescence (Chen et al., 2014). *NCED3* and *AAO3* are key factors regulating the ABA biosynthesis pathway in plants (Barrero et al., 2006). *NCED3*, which catalyzes the cleavage reaction of 9-*cis*-xanthophylls, is a rate-limiting factor, while *AAO3* catalyzes the final step in the ABA biosynthesis pathway (Melhorn et al., 2008). Exogenously applied ABA reduced chlorophyll content in detached leaves, and increased the transcript level of *SGR1* in *Arabidopsis* (Yang et al., 2014). Methyl jasmonate (MeJA) and salicylic acid (SA) also have effects on both chlorophyll degradation and the expression of genes related to senescence (He et al., 2002; Chen et al., 2014; Liu et al., 2015).

Zoysiagrass (*Zoysia japonica*), a common warm-season grass, is well adapted for home lawns and sport fields in the transitional and warm climatic regions, because of its excellent traffic tolerance, low maintenance requirements, and high coverage (Patton and Reicher, 2007). Expanding the use of zoysiagrass is important to make football fields, golf courses, and home lawns more sustainable and environment-friendly (Patton et al., 2007). Nevertheless, a barrier to spread its use more widely is the de-greening phenotype that occurs during harsh seasons. To date, limited genetic resources are available for zoysiagrass (Wei et al., 2015; Xie et al., 2015). Further investigation of the possible genetic factors regulating chlorophyll degradation and senescence would contribute to the application of zoysiagrass. In this study, *ZjSGR* was isolated and characterized from *Z. japonica*. Furthermore, the subcellular

localization and expression patterns were studied. The results showed that *ZjSGR* is crucial to chlorophyll degradation and senescence in plants. This information could aid further attempts to explore the possible genetic mechanism underlying chlorophyll degradation and senescence in zoysiagrass.

MATERIAL AND METHODS

Plant materials

Z. japonica seeded cultivars ‘Zenith’, purchased from Hancock seed company (Hancock, Dade, Florida, USA), were grown in a greenhouse maintained at average day/night temperatures of 28/25°C. *Nicotiana benthamiana* plants were grown in a growth chamber at 22°C with 16-h photoperiod. The plants were fertilized with half-strength Hoagland’s solution (Hoagland and Arnon, 1950) every week.

Isolation of *ZjSGR*

Total RNA was extracted from 3-month-old ‘Zenith’ leaves using the Trizol (Tiangen Biotech, Beijing, China) method and then pretreated extensively with an RNase-free DNase (Tiangen Biotech, Beijing, China) to eliminate genomic DNA before use. The 5'-full-length sequence was obtained through 5'-rapid amplification of cDNA ends (RACE) according to the manufacturer instructions (SMARTer RACE 5' Kit, Takara, Dailian, China). The 5'-RACE primer was designed based on a fragment from the *ZjSGR* cDNA sequence (GenBank accession No. AY850154). Purified PCR products were cloned into a pEASY-T1 simple vector (TransGen Biotech, Beijing, China), and were then sequenced at the Beijing Genomics Institute (Beijing, China). Based on the sequence obtained, a pair of gene-specific primers, *ZjSGR*-F and *ZjSGR*-R (Table 1), were designed to amplify the full-length *ZjSGR* cDNA and gDNA. Intron-exon structure of *ZjSGR* was constructed by manual alignment of the cDNA and gDNA sequences.

Table 1. Primers used in this study.

Primer name	Sequence (5'-3')	Primer purpose
5'-RACE	CGGTTGTACCACCCTGCAGCTGCGCG	5'-RACE-PCR
<i>ZjSGR</i> -F	TTCTGGTCCGCTCCCAGGAGAAGGG	RT-PCR of <i>ZjSGR</i> cDNA and gDNA
<i>ZjSGR</i> -R	TGTCATCACCGGTCCCGTGTACACC	
3302Y-F	cctCCATGGATGGCTGCGGCCATT	Constructing subcellular localization vector of <i>ZjSGR</i>
3302Y-R	cctACGCGTCTGCTGCGTCTGGCC	
qPCR-F	CGTCCACTGCCACATCTCCG	qRT-PCR analysis of <i>ZjSGR</i>
qPCR-R	CGAACGCCTTCAGCACACA	
SAG113-F	GGTGCGAACTTCAGACTCCA	qRT-PCR analysis of <i>NtSAG113</i>
SAG113-R	ATTCATCTGGTCGATCCGGC	
NCED3-F	AGCTTTGGGTCGTCCTGTTT	qRT-PCR analysis of <i>NtNCED3</i>
NCED3-R	AAACCAGCGTTTGCAACTCC	
AAO3-F	AgTgCCCCgAgTATgTAggT	qRT-PCR analysis of <i>NtAAO3</i>
AAO3-R	TCAgICTTTCggTTgACgCA	
ACT-F	GGTCCTCTTCCAGCCATCCTTC	qRT-PCR of <i>Zoysia Actin</i> gene used as internal control
ACT-R	GTGCAAGGGCAGTGATCTCCTT	
NtACT-F	AGGTTACGCCCTTCTCAT	qRT-PCR <i>Actin</i> of <i>N. benthamiana</i> used as internal control
NtACT-R	GGACAATTTCCCGTTCAGC	

Bioinformatics analysis

The amino acid sequence of *ZjSGR* was predicted from the cDNA sequence, using the DNAMAN software (v. 7.0). Homologues were screened using BLAST in the NCBI database. A phylogenetic tree was built using MEGA v. 5.0 with a neighbor-joining method (Tamura et al., 2011). A bootstrap analysis was performed with 1000 replicates excluding positions with gaps. The theoretical isoelectric points (pI) and molecular weights (MW) were predicted using the compute pI/MW tool (http://web.expasy.org/compute_pi/). Potential signal peptide cleavage sites were identified using the SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>) (Petersen et al., 2011). To predict the subcellular localization of *ZjSGR*, the protein sequence was analyzed using the online ProtComp v. 9.0 program (<http://www.softberry.com>).

Subcellular localization of *ZjSGR*

To examine the subcellular localization of *ZjSGR*, a pair of primers, 3302Y-F and 3302Y-R (Table 1), were designed to amplify the coding sequence of the *ZjSGR* gene. The PCR product was digested with *Nco*I and *Mlu*I, and inserted into a 3302Y vector, which was digested with the same enzymes. The *ZjSGR*-YFP fusion construct was then transformed into *N. benthamiana* (Yang et al., 2000). Following a 48 h dark treatment, the leaf epidermal cells were visualized using a laser confocal scanning microscope (Leica SP-5; Leica, Mannheim, Germany).

Quantitative real-time PCR

In order to investigate the expression pattern of *ZjSGR* in zoysiagrass, quantitative real-time polymerase chain reactions (qRT-PCR) were carried out both in leaves at different senescent stages and in other tissues (root, stem, and leaf). The expression profiles of *ZjSGR* were determined in 3-month-old zoysiagrass induced by a time course of 24 h with 10 μ M ABA, 10 μ M MeJA, or 0.5 mM SA. For the transient overexpression pattern of *NtSAG113*, *NtAAO3*, and *NtNCED3*, the leaves were ground with liquid nitrogen at 3 d after infiltration. The *ZjSGR*-specific primers used in the qRT-PCR were qPCR-F and qPCR-R (Table 1). The qRT-PCR was performed in 96-well blocks with an RT-PCR system (CFX Connect, BIO-RAD, Hercules, California, USA) using an UltraSYBR Mixture (CoWin, Beijing, China) at a volume of 25 μ L. The two-step thermal cycling profile used was 15 s at 95°C and 1 min at 68°C. The zoysiagrass *Actin* gene (GenBank accession No. GU290546) and tobacco *Actin* gene (GenBank accession No. AB158612), respectively, were used as controls to normalize the amount of cDNA. The relative expression was calculated using the comparative $\Delta\Delta$ threshold cycle method (Chao et al., 2009). All results are presented as the means of at least three independent RNA extractions (including three technical replicates) with corresponding standard deviations (SD).

Transient overexpression in *N. benthamiana*

Agrobacterium infiltration was performed in the leaves of 1-month-old *N. benthamiana* plants with *Agrobacterium* strain EHA105 carrying the *ZjSGR*-YFP fusion construct and 3302Y empty vector (negative control), based on Yang's method with some modifications (Yang et al., 2000). Symptom development and gene expressions were monitored from day three after infiltration.

Endogenous ABA measurement

The ABA in tobacco leaves was extracted and purified following the protocol of Man et al. (2011) with minor modifications. Frozen leaf tissues (100 mg) were ground to powder using a mortar and pestle in liquid nitrogen. The sample was extracted in 1 mL sodium phosphate buffer (50 mM, pH 7.0) containing 0.02% sodium diethyldithiocarbamate as an antioxidant. An indirect enzyme-linked immunosorbent assay was used for ABA determination, using the ABA-ELISA kit (Shanghai Baiwo Company, Shanghai, China) according to the manufacturer instructions. The ABA contents were then calculated on the basis of a prepared standard curve and expressed as ng/g fresh weight.

RESULTS

Isolation of the *ZjSGR* gene and bioinformatics analysis

Both cDNA and gDNA sequences were submitted to the NCBI database, and the accession Nos are KP148819 and KP148820, respectively. The full-length cDNA of the *ZjSGR* gene is 831 bp in length. The analysis of the amino acid sequence revealed that the *ZjSGR* protein contains 276 amino acids and belonged to the stay-green superfamily (Figure 1A). The theoretical pI is 8.70 and molecular mass is 31.07 kD. No potential signal peptide was identified in the *ZjSGR* protein, based on the SignalP 4.1 Server prediction. The comparison of gDNA with cDNA showed that *ZjSGR* has three exons and that the translation initiation codon is located within the first exon (Figure 1B). The predicted SGR proteins used for phylogenetic analysis were collected from several species from GenBank. The GenBank accession Nos of *ZjSGR* and its homologs in other plants are as follows: *ZjSGR*, AKA59741; *ZmSGR1*, AAW82956; *SbSGR*, AAW82958; *ZmSGR2*, AAW82957; *BeSGR*, ADK56113; *FaNYE1*, ADV57294; *HvSGR*, AAW82955; *CaSGR*, ABX82698; *SISGR1*, AAY98500; *LcSGR*, AKA88530; *GmSGR2*, AAW82960; *GmSGR1*, AAW82959; *MsSGR*, AEE00202; *MtSGR*, AEE00201. The phylogenetic tree was divided into two clades, one belonging to monocotyledonous species and the other belonging to dicotyledonous species. The phylogenetic comparison showed that *ZjSGR* is closest related to *ZmSGR1* (Figure 1C).

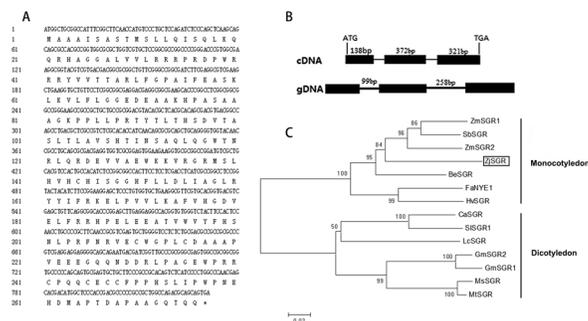


Figure 1. Sequence and phylogenetic analysis of *ZjSGR*. **A.** The *ZjSGR* sequence and its encoded protein. **B.** The structure of the *ZjSGR* cDNA and gDNA. Black boxes indicate exons and black lines indicate introns. The translational start codon (ATG) and stop codon (TGA) are marked. **C.** Phylogenetic analysis of the *ZjSGR* protein and its orthologs. Bootstrap values are provided to indicate the reliability at each node. The scale bar corresponds to estimated amino acid substitutions per site.

The expression pattern of *ZjSGR*

To determine the expression pattern of the *ZjSGR* gene in zoysiagrass, qRT-PCR was carried out. The transcript level of *ZjSGR* was detected in all tissues including roots, stems, and leaves, and a much higher expression level was detected in leaves (Figure 2A). The transcript level was also found to be higher in senescent leaves than in young leaves (Figure 2B). When zoysiagrass was exposed to 10 μ M ABA or 10 μ M MeJA, expression levels of *ZjSGR* were obviously increased at 12 and 3 h respectively (Figure 2C-D). However, the mRNA level of *ZjSGR* was suppressed drastically following 24 h treatment of 0.5 mM SA (Figure 2E). After 24 h of total darkness treatment, the *ZjSGR* expression was found to be strongly up-regulated (Figure 2F).

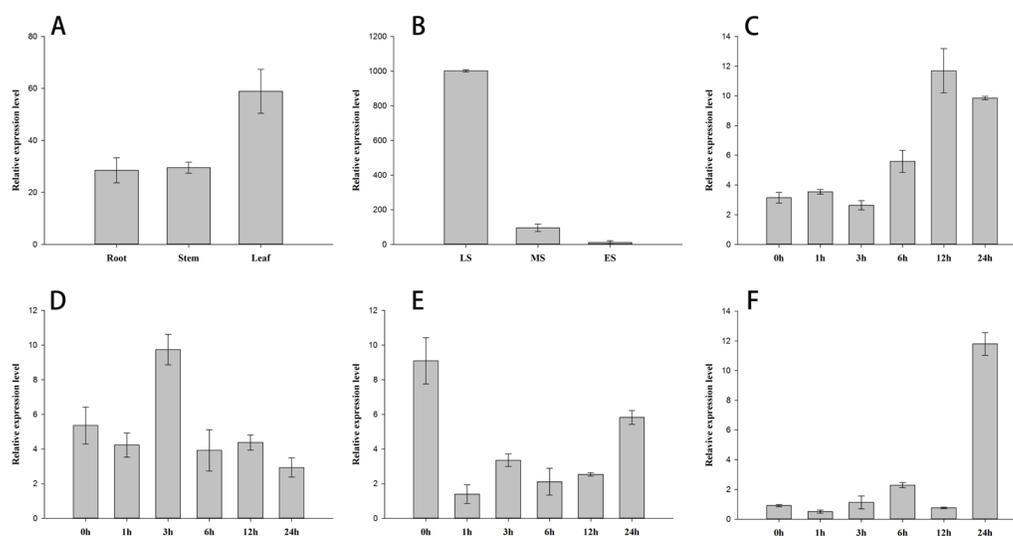


Figure 2. Expression analysis of *ZjSGR*. **A.** Expression levels of *ZjSGR* in root, stem, and leaf of the zoysiagrass. **B.** Expression levels of *ZjSGR* in leaves at different senescence stages. LS, MS, and NS represents serious, middle, and slight senescence, respectively. **C.-E.** *ZjSGR* expression levels treated by 10 μ M ABA, 10 μ M MeJA, and 0.5 mM SA. **F.** Expression level of *ZjSGR* during 24 h of continuous dark treatment.

Subcellular localization of *ZjSGR*

The results predicted using the ProtComp v. 9.0 program demonstrated that the *ZjSGR* protein is most likely localized to the chloroplasts (with a prediction score of approximately 9.3). To determine the exact subcellular localization of the *ZjSGR* protein, the construct of *ZjSGR*-YFP driven by 35S promoter was applied to transform *N. benthamiana* leaf cells. The results showed that *ZjSGR* was localized to the chloroplast (Figure 3).

Transient overexpression of *ZjSGR* accelerate chlorophyll degradation and senescence in *N. benthamiana*

To investigate the functional role of *ZjSGR*, a transient overexpression assay was performed. An *Agrobacterium*-mediated transient transformation was conducted on the leaves

of 1-month-old *N. benthamiana* with recombinant agrobacteria EHA105 carrying either 35S_{pro}: *ZjSGR* plasmid construct or negative control. The results showed that the green leaf spots infiltrated by 35S_{pro}: *ZjSGR* began to turn yellow at 3 d after infiltration and continued to spread widely (Figure 4). In contrast, those infiltrated by negative control remained green. Four of the 1-month-old plants were used for each infiltration and showed similar results. The qRT-PCR results showed that transient overexpression of *ZjSGR* up-regulated the expression of *NtSAG113*, *NtAAO3*, and *NtNCED3* (Figure 5A-D). Compared with the controls, the *NtSAG113*, *NtAAO3*, and *NtNCED3* expression levels in the transgenic leaves were increased to approximately 40, 4, and 6-fold, respectively. This suggests that transient overexpression of *ZjSGR* accelerated leaf senescence by positively regulating senescence associated genes and triggering ABA biosynthesis. To verify this assumption, the endogenous ABA contents was measured. Our results showed that the ABA contents increased 16.67% in the *ZjSGR*-overexpressing leaves (Figure 5E), which confirmed our assumption.

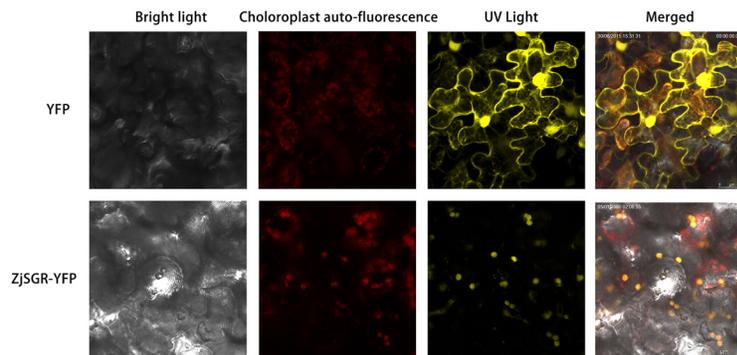


Figure 3. Subcellular localization of *ZjSGR* in *Nicotiana benthamiana* leaf cells. Fluorescence due to the *ZjSGR*-YFP fusion protein was strongly detected in the chloroplasts.



Figure 4. Transient expression of *ZjSGR* in *Nicotiana benthamiana*. **A.** Plant phenotypes of *N. benthamiana* infiltrated by recombinant agrobacteria containing 35S_{pro}: *ZjSGR*-YFP (left plant) or YFP (right plant) in the 3302Y vector at 3 d after infiltration. **B.** The infiltrated leaf phenotypes of *N. benthamiana* as shown in **A.**

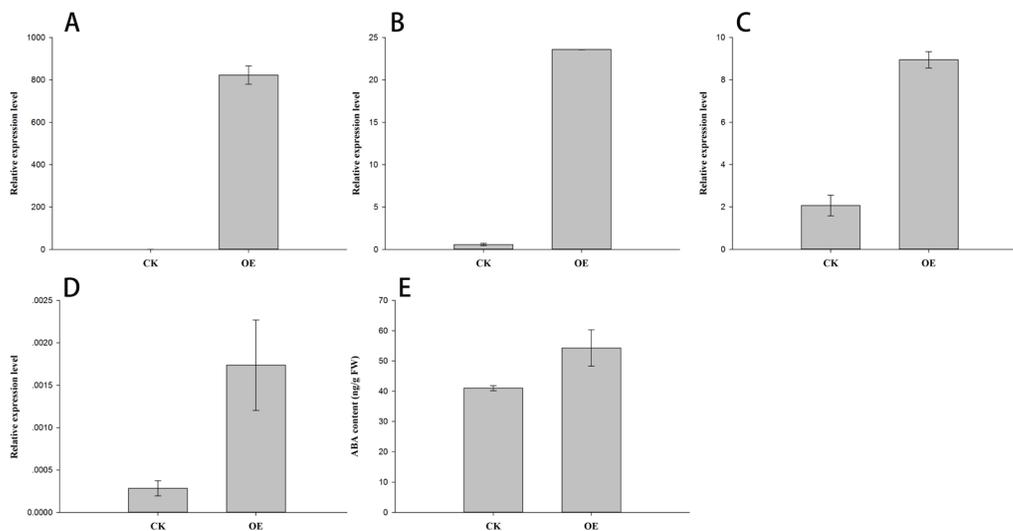


Figure 5. Gene expressions and ABA contents in *Nicotiana benthamiana* leaves expressing *ZjSGR* and negative control. **A.-D.** Relative expression levels of *ZjSGR*, *NtSAG113*, *NtAAO3*, and *NtNCED3*, respectively. **E.** ABA contents in CK and OE tobacco.

DISCUSSION

Although green period and lawn color improvements are two major focuses in zoysiagrass breeding, the regulatory mechanisms of chlorophyll degradation and senescence have not yet been elucidated. Therefore, a study designed to investigate the molecular mechanism of chlorophyll degradation and senescence of *Z. japonica* was performed. Firstly, a new senescence-induced gene named *ZjSGR* was isolated from *Z. japonica*. The coding sequence was found to be 831 bp in length, encoding for 276 amino acids. A phylogenetic analysis revealed that *ZjSGR* is closest related to the homologue *ZmSGR1*. Subcellular localization analysis showed that *ZjSGR* is localized to the chloroplasts, which is consistent with the SGRs in *A. thaliana* and *Oryza sativa* (Park et al., 2007; Ren et al., 2007).

Previous studies have demonstrated that the expression levels of *SGR* are correlated with the severity of leaf senescence (Wei et al., 2011). A similar expression pattern was observed also in this study. The expression level of *ZjSGR* was found to be nearly 50 times higher in severely senescent leaves than in young leaves. The *ZjSGR* expression level was also higher in leaves than in roots and stems. In addition, *ZjSGR* could be strongly induced by dark treatment. After 24 h of continuous dark treatment, its expression level was up to 12 times higher than the baseline level, indicating that *ZjSGR* was positively regulated by darkness. Exogenous ABA and MeJA have been reported to accelerate chlorophyll degradation, and up-regulate the expression of some *SAGs* (Kim et al., 2011; Zhang et al., 2012). As expected, the expression level of *ZjSGR* was up-regulated by natural senescence, and by exogenous ABA and MeJA. Together these results suggest that *ZjSGR* may play an essential role in chlorophyll degradation and senescence processes in *Z. japonica*. Interestingly, *ZjSGR* was strongly suppressed by SA in our study. Since SA has recently been reported to play a dual role in promoting senescence and cell death (Schippers et al., 2015), further studies are required to

investigate the mechanism. This is the first study of differential expression of the *ZjSGR* gene in different tissues and induced by hormone treatments.

Transient gene expression assays provide an efficient and versatile tool for studying foreign gene expression (Yang et al., 2000; Bhaskar et al., 2009). In addition, transient gene expression is not biased by position effects, which often occur in stable transformation (Lee and Yang, 2006). In this study, the spreading of yellowish leaf spots suggests that *ZjSGR* could accelerate chlorophyll degradation in tobacco leaves. This is consistent with the study of transient overexpression of rice SGR in tobacco (Park et al., 2007). In our study, *NtSAG113* expression was significantly up-regulated in transgenic tobacco. *SAG113* expression is regulated in a senescence-specific mode (Zhang et al., 2012). Our data provided evidence that overexpression of *ZjSGR* could accelerate leaf senescence. ABA is essential in triggering leaf senescence (Xue-Xuan et al., 2010). *AAO3* and *NCED3* are key genes regulating the ABA biosynthesis in plants (Barrero et al., 2006). The activated ABA biosynthesis, relating to the increased ABA contents and transcript abundance of *NtAAO3* and *NtNCED3*, confirmed the role of *ZjSGR* in leaf senescence. However, in light of the observed gene expression levels, *NtSAG113* appeared more important compared to *NtAAO3* and *NtNCED3*, for *ZjSGR* to promote leaf senescence.

In summary, our results suggest that *ZjSGR* is a chloroplast localized protein, which can be up-regulated by natural senescence, darkness, or ABA treatment. *ZjSGR* could accelerate the progresses of chlorophyll degradation and leaf senescence. Therefore, *ZjSGR* could be a valuable gene for improving the de-greening phenotype of *Z. japonica* by genetic engineering.

Conflicts of interest

The authors declare no conflict of interest.

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