# Silencing of the *LeSGR1* gene in tomato inhibits chlorophyll degradation and exhibits a stay-green phenotype

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## Abstract

The full-length cDNA of *LeSGR1* was cloned from tomato by RT-PCR and RACE. The cDNA encoded a protein of 272 amino acid residues and was deposited in GenBank (accession No. DQ100158). Northern analysis suggests that *LeSGR1* gene specifically expresses in senescent leaves and mature fruits of tomatoes. Desiccation and flooding induce the expression of *LeSGR1* in tomato leaves and stems. Both in ethylene-insensitive mutants (*Nr*) and *ripening inhibitor* mutants (*rin*), the expression of *LeSGR1* is markedly decreased compared with that in the wild type. Alignment of the nucleotide sequence of *SGR1* cloned from the tomato *green flesh* (*gf*) mutant with that from the wild type tomato shows a single nucleotide change leading to an amino acid substitution in *gf* mutant. Furthermore, *LeSGR1* gene silencing by RNA interference results in inhibited chlorophyll degradation similar to the phenotype in *gf* mutant. Thus, we conclude that *LeSGR1* is crucial to chlorophyll degradation and the mutation of SGR1 protein might be responsible for *gf* tomato properties.

*Additional key words*: desiccation, flooding, *Lycopersicon esculentum*, mutants, senescence.

## Introduction

Yellowing is the characteristic symptom of leaf senescence in most botanical species. It is a course in which chlorophylls (Chl) degrade, other chromophylls such as xanthophylls accumulate, and chloroplasts in leaves turn into chromoplasts. During the last few years, the basic steps of the Chl degradation pathway have been revealed (Hörtensteiner 2006). In the first step Chl *a* is converted by chlorophyllase (Chlase) into chlorophyllide *a*. In the next step, an unknown metal-chelating substance (MCS) removes magnesium and produces pheophorbide *a* (Pheide *a*). Pheide *a* is then oxidized into red chlorophyll catabolite (RCC), with its porphyrin-macrocycle cleft in the presence of pheophorbide *a* oxygenase (P*a*O), and the RCC is immediately converted into primary fluorescent chlorophyll catabolite (pFCC) by red chlorophyll catabolite reductase (RCCR). The pFCC undergoes several modifications such as demethylation and hydroxylation depending on the species of plants, and it is changed to fluorescent chlorophyll catabolites (FCCs). Some of the FCCs are exported from gerontoplast to vacuole by an ATP-dependent translocator in the envelope membrane. Finally, FCCs are nonenzymically tautomerized to the respective nonfluorescent chlorophyll catabolites (NCCs) in acidic environment inside the vacuole. The degradation of Chl *b*, starts with conversion into Chl *a*, then goes through the same journey as Chl *a* does (Hörtensteiner 2006). Chlorophyll *b* reductase and hydroxymethyl chlorophyll *a* reductase control the reduction process of Chl *b* into Chl *a*.

The loss of Chl has been deemed as one of the necessary events in leaf senescence for many years. However, this long-held conception has been challenged since the discovery of stay-green (SGR) mutants in *Festuca pratensis* (Thomas 1987), *Phaseolus vulgaris*

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*Abbreviations*: $AC^{++}$ - Ailsa Craig; B - breaker; Chl - chlorophyll; dpa - days post anthesis; *gf* - *green flesh*; IMG - immature green; MG - mature green; ML - mature leaf; *Nr* - *never ripe*; ORF - open reading frame; RACE - rapid amplification of cDNA ends; *rin* - *ripening inhibitor*; RT PCR - reverse transcriptase polymerase chain reaction; SL - senescent leaf; UTR - untranslated region; YL - young leaf.
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(Hardwick 1979), *Glycine max* (Woodworth 1921), *Sorghum vulgare* (Vietor *et al.* 1989), *Oryza sativa* (Mondal and Choudhuri 1985), *Capsicum annuum* (Smith 1950), *Lycopersicon esculentum* (Kerr 1956), *etc*. Stay-green refers to foliar senescence that occurs without significant Chl degradation. It has been found that different stay-green mutants showed similar stay-green presentation. Nevertheless, there were many differences in their response to phytohormones (Thomas and Stoddart 1975, Wittenbach 1977), in photosynthetic capacity (Hilditch *et al.* 1986, Guiamét *et al.* 1990), stability of photosynthesis related enzymes or proteins (Crafts-Brandner *et al.* 1984), tissue specificity in expression of mutant characteristics (Ambler *et al.* 1987) and genetic mechanism mutants (Thomas and Smart 1993). The reason for that is different genetic basis. A typical case was the non-yellowing mutant bf993 found by Thomas's group in 1969 (Shen 2001). They demonstrated that bf993 was a single allele recessive mutant, and designated it as SID (senescence-induced degradation), a nuclear gene responsible for controlling the breakdown of Chl porphyrin-macrocycle. Thomas and Howarth (2000) classified stay-green events in leaf senescence into five categories by Chl content and photosynthetic capacity. Specifically, type A loses pigment and function at the normal speed after a delay in the start of leaf senescence, *e.g*. corn mutant fs854 (Crafts-Brandner *et al.* 1984); type B loses pigment and function simultaneously, but at a lower speed than a standard genotype, *e.g*. *Sorghum vulgare* mutant (Ambler *et al.* 1987, Sanchez *et al.* 2002); in type C, functional senescence undergoes normally, but Chl breakdown is hindered, *e.g*. *Festuca pratensis* mutant bf993 (Hörtensteiner 2006, Hilditch *et al.* 1986, Thomas *et al.* 1999); in type D, photosynthesis stops but Chl content remains unchanged when the leaf is killed by freezing, boiling or drying; and type E has a normal photosynthetic capacity but a higher absolute Chl content. The types A and B maintain both leaf greenness and photosynthetic activity much longer and thus are defined as functional stay-green; whereas the types C, D, and E have persistent leaf greenness without sustaining photosynthetic competence during senescence, and are classified as nonfunctional stay-green.

The physiological, cytological, biochemical and genetic characteristics of stay-green mutants have been understood well. With the development of molecular biology, however, the researchers start to pay more attention to the molecular mechanism of the stay-green phenotype. Cha *et al.* (2002) isolated a stay-green mutant from the glutinous japonica rice Hwacheong-wx through N-methyl-N-nitrosourea mutagenesis. Research on this mutant revealed that the stay-green phenotype was controlled by a single recessive nuclear gene which was denoted by *SGR1* and located on the long arm of chromosome 9 between RFLP markers RG662 and C985. In 2007, Armstead's group silenced the *Arabidopsis SGR* gene through RNA interference (RNAi), resulting in plants with a stay-green phenotype equivalent to the original stay-green *Festuca pratensis* plants (Armstead *et al*. 2007). *SGR* genes were also isolated from other high green plants and deposited at GenBank, such as, *Arabidopsis thaliana* (GenBank accession no. AY850161), *Hordeum vulgare* (GenBank accession no. AY850135), *Zea mays* (GenBank accession no. AY850138), *Oryza sativa* (GenBank accession no. AY850134), *Zoysia japonica* (GenBank accession no. AY850154), *Sorghum bicolor* (GenBank accession no. AY850140) and *Glycine max* (GenBank accession no. AY850141). *SGR* gene is a kind of nuclear gene, encoding an ancient protein containing a putative chloroplast transit peptide, highly induced by senescence signal. It is not clear so far how this gene functions in Chl degradation.

The *green flesh* (*gf*) mutant of tomatoes was found as early as in 1955 (Kerr 1956). In this mutant, Chl degradation is defective and normal chloroplast-chromoplast transition is altered, giving rise to a rusty red fruit and dark green senescent leaf color (Cheung *et al.* 1993). The mutation is controlled by a single recessive gene, which is assigned to chromosome 8 in the morphological mutants map (Kerr 1957). In order to find out whether this single recessive nuclear gene is homologous to the *SGR* genes reported in *Arabidopsis thaliana* and other stay-green mutant, we cloned the *LeSGR1* (tomato *SGR1*) gene from the *gf* mutant and the wild type tomato, and analyzed the expression patterns of *LeSGR1*. Through DNA sequence alignment between the *gf* mutant and the wild type tomato *SGR1*, we found a single nucleotide substitution in coding domain sequence (CDS) in the *gf* mutant. This nucleotide substitution led to a single amino acid change. We wondered whether this missense mutation of SGR1 protein is responsible for *gf* tomato mutation. *LeSGR1* RNA interference (RNAi) expression vector was constructed and transformed into the wild type tomato. The *LeSGR1*-silenced transgenic plants showed a stay-green phenotype.

## Materials and methods

**Plants:** The plants used in our study were *Lycopersicon esculentum* Mill. cv. Ailsa Craig ($AC^{++}$) and tomato mutants *never ripe* (*Nr*), *ripening inhibitor* (*rin*), and *green flesh* (*gf*), which were a kind gift from Prof. Donald Grierson, Nottingham University. All tomato plants were near-isogenic lines and grew in 24 cm diameter pots at an average temperature of 25 °C in a growth room with supplementary lighting (16-h photoperiod) at irradiance of 400 μmol $m^{-2}$ $s^{-1}$. The pots were filled with a mixture of clay-loam soil, compost, riversand and plant ash and plants were watered daily and fed with fertilizer (N : P : K, 5:1:2.8; *Fuling Chemical Industry Company*, Chongqing, China). Flowers were tagged at anthesis and fruit development recorded as days post-anthesis (dpa).

Immature green (IMG) fruits were defined as those about 20 dpa after anthesis. Mature green (MG) fruits were defined at 35 dpa and were green and shiny with no obvious ripening-associated color change. Breaker (B) fruits were defined as showing the first signs of ripening-associated color change from green to yellow. Fruits of subsequent ripening stages were defined in days post-breaker so that B+4 fruits were orange/red in color. All plant samples for the preparation of total RNA were taken at the same time of day, frozen in liquid nitrogen and stored at -70 °C until required.

**Chemicals:** All chemicals were of at least analytical-reagent grade. Formaldehyde, alcohol, acetone were purchased from *Chuandong Chemical Reagent Company* (Chongqing, China). For Northern blot hybridization, agarose, formamide, SDS, LiCl were purchased from *Dingguo Biotechnology Company* (Beijing, China). The restriction enzymes were purchased from *Takara* (Dalian, China). Primers were synthesized by *Invitrogen* (Shanghai, China).

**Plant treatments:** For water deficit stress experiments, well hydrated 1-month-old plants grown in 9 cm pots in compost, were cut at the base of roots. Half of the plant materials were subjected to water deficit stress treatment in a dry beaker for 8 h. The other plants were put in another beaker with water to immerge cut surface and were taken as the control. Leaf and stem samples were taken from the treated and control tomato plants.

For flooding treatment, 1-month-old plants grown in pots in compost were placed in a plastic box with water to immerge roots. Leaves were harvested in 1, 3 and 5 d after flooding. Control plants were watered normally.

For senescence studies, mature leaves were detached from transgenic tomato plants and placed on three layers of wet *Whatmann No. 1* filter paper in 14-cm Petri dishes. The dishes were incubated in the dark at 23 °C for 7 d. Wild type ($AC^{++}$) leaf was used as control.

**Gene isolation:** Total RNA was isolated from fruit pericarp as previously described (Chen *et al.* 2004). Then it was used as the template to synthesize the first strand cDNA through reverse transcription polymerase chain reaction (RT-PCR) with primer Z9 (5'TGGATCCGC TGAAACTCTAGGTTTTTTTTTTTTTTTTTT3'). Degenerate upstream primer F1 (5'ATGTC(G/T)CT(T/G) CACGT(T/G)CA(T/C)TG(T/C)CACAT3') was designed according to the conserved sequences in *Arabidopsis thaliana* (AY850161), *Sorghum bicolor* (AY850140), *Zea mays* (AY850138), *Hordeum vulgare* (AY850135), *Oryza sativa* (AY850134) and *Glycine max* (AY850141) SGRs, The cDNA fragment of *LeSGR1* was amplified by polymerase chain reaction (PCR) using *Prime STARTM HS* DNA polymerase (*Takara*, Dailian, China) with primers F1 and Z10 (5'TGGATCCGCTGAA ACT CTAGGT3'), following a protocol composed of 3 min denaturation reaction at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 52 °C and 60 s at 72 °C, and finally extended by 10 min at 72 °C. The amplified product was separated and purified by 1.5 % agarose gel electrophoresis, tailed by using DNA *A-Tailing* kit (*Takara*, Dailian, China), linked with *pMD18-T* vector (*Takara*, Dailian, China) and transformed into *Escherichia coli* JM109. Positive clones were picked out and confirmed by sequencing (*Invitrogen*, Shanghai, China). The 5'-RACE (rapid amplification of cDNA ends) PCR was performed according to the manufacturer's instructions (5'-full RACE core set, *Takara*, Dailian, China), and the 5'end of the *LeSGR1* was cloned and sequenced. The *LeSGR1* full-length cDNA was isolated by combining the above two parts of fragments.

**Northern blot hybridization:** 4 g fresh tissue was put in liquid nitrogen in a mortar and grinded quickly to a fine powder. Total RNA was extracted according to the method described by Chen *et al.* (2004) and sugars and DNA were removed by differential precipitation of the RNA by 4 M LiCl at 20 °C for 1 h. RNA concentration and purity were quantified by *PerkinElmer lambda 900* UV/VIS spectrometer (*PerkinElmer*, Waltham, MA, USA) (Wang *et al.* 2008). Then total RNA was formamide denatured, and 20 µg samples were fractionated in 1 % (m/v) agarose gels containing 3 % (v/v) formaldehyde. RNA was capillary blotted onto *Hybond N* (*Amersham International*, Little Chalfont, UK) membranes which were then pre-hybridised and hybridised to $^{32}$P-labelled probes generated from *LeSGR1* cDNA using the rediprime labelling system (*Amersham International*), as previously described by Chen *et al.* (2004). After hybridization, membranes were washed in 0.2× SSPE, 0.1 % (m/v) sodiumdodecyl-sulphate at 42 °C and autoradiographed.

**Construction of transgene and plant transformation:** To construct *LeSGR1* RNAi (RNA interference, RNAi)

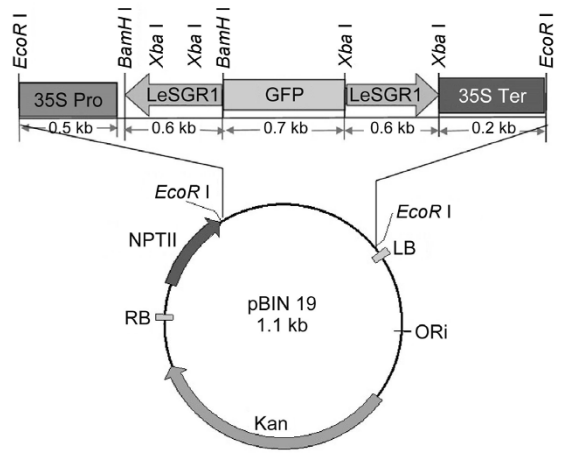


Fig. 1. The construct of RNAi vector for *LeSGR1* gene silencing. The *LeSGR1* gene-specific sequence in the antisense and sense orientations were linked with a *GFP* gene fragment and as a transcriptional unit for hairpin RNA expression which was controlled by the CaMV 35S promoter and terminated by the 35S terminator.

expression vector, a 600-bp segment of the *LeSGR1* cDNA was amplified using primers with *Bam*H I and *Xba* I restriction sites at the 5'end, then digested with *Bam*H I and *Xba* I respectively, and linked into the pDHG 5.2 plasmid at *Xba* I restriction site in the sense orientation and at *Bam*H I restriction site in the antisense orientation. After that, digested with *Eco*R I, the double-stranded (ds) RNA expression unit, containing the cauliflower mosaic virus (CaMV) 35S promoter, *LeSGR1* fragment in antisense orientation, GFP gene fragment, LeSGR1 fragment in sense orientation and 35S terminator, was purified and subcloned into the plant binary vector pBIN 19 (*Takara*) to yield RNAi vector for *LeSGR1* gene silencing (Fig. 1). The construct was confirmed by restriction digest analysis and then by sequencing. After transferred to *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (Bird *et al.* 1988), the construct was used to transform $AC^{++}$ tomato cotyledon explants (Chen *et al.* 2001). Transgenic plants that rooted on kanamycin were transferred to composts and grown as described above.

**Semi-quantitative RT-PCR:** To examine the silencing effect of *LeSGR1* gene in the transgenic plants, total RNA was extracted from tomato fruits and DNA was removed as described above. The first strand cDNA was synthesized from 3 μg of total RNA using primer Z9. Then the first strand cDNA mixture was used as template for semi-quantitative PCR analysis. The tomato *actin* gene (GenBank accession No. BT012695) was used as a reference for determining the expression level of the *LeSGR1*. The *LeSGR1* gene was amplified as follows: one cycle of 3 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 25 s at 72 °C, and one cycle of 10 min at 72 °C. For *actin*, the PCR procedure was one cycle of 3 min at 94 °C, 28 cycles of 30 s at 94 °C, 30 s at 55 °C and 20 s at 72 °C, and one cycle of 10 min at 72 °C. The amplification products were run on a 1.5 % agarose gel, and determined by gel imaging system.

**Chlorophyll extraction and determination:** Total Chl was extracted and its content was determined as described by Becker (1994). Samples mashed in liquid nitrogen were extracted by ethanol : acetone (2:1,v/v) for 48 h in the dark. The extract was centrifuged at 14 000 *g* for 5 min and the absorbance of supernatant was measured at 645 and 663 nm. Three independent experiments were performed for each sample.

## Results

**Gene isolation and sequence analysis of wild-type and *gf* mutant alleles:** The full-length cDNA of *LeSGR1* is 1206 bp in size, containing a 145 bp 5'UTR, a 259 bp 3'UTR and an 816 bp ORF. The cDNA encodes a protein of 272 amino acid residues with a calculated molecular mass of 30 kD and an isoelectric point of 8.74. By *BLAST* and multiple alignment analysis (*DANMAN 6.0*), the deduced amino acid sequence shares 70.3, 56.5, 56.8, 58.0, 60.1 and 71.1 % identity to AtSGR (*Arabidopsis thaliana* SGR), SbSGR (*Sorghum bicolor* SGR), ZmSGR (*Zea mays* SGR), HvSGR (*Hordeum vulgare* SGR), OsSGR (*Oryza sativa* SGR) and GmSGR (*Glycine max* SGR), respectively (Fig. 2). Sequence analysis suggests it belongs to SGR family. The sequence data were deposited in GenBank under accession number DQ100158.

Four phosphorylation sites (threonine 82, serine 202,

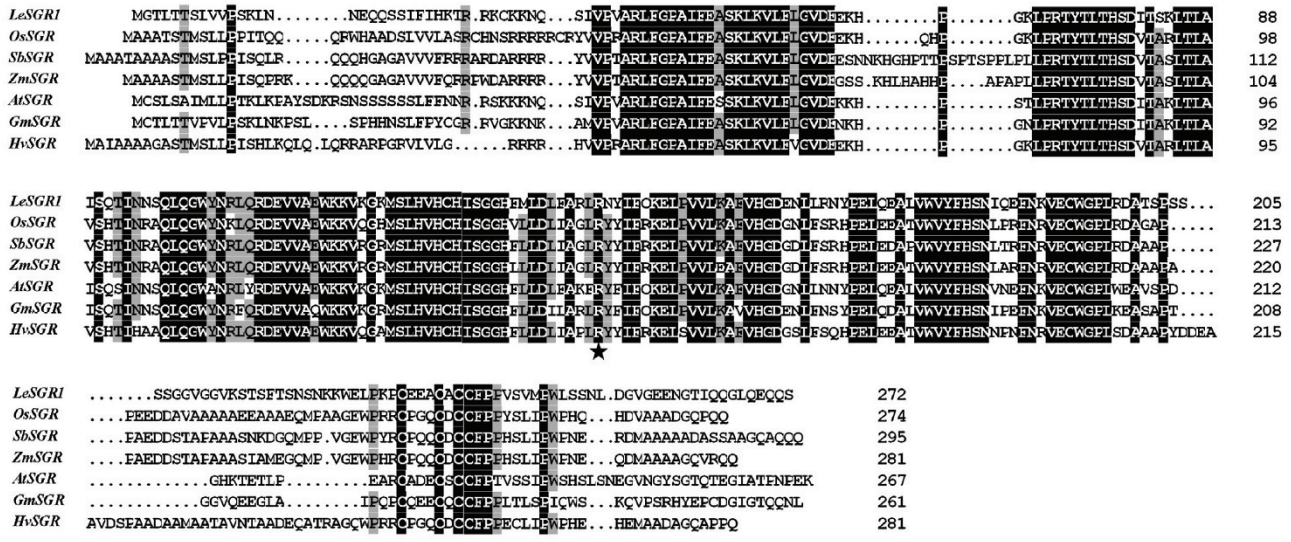


Fig. 2. Amino acid alignment of SGR proteins. Among the seven SGR proteins (LeSGR1, AtSGR, SbSGR, ZjSGR, HvSGR, OsSGR and GmSGR), the identical amino acids are highlighted in black and similar amino acids are highlighted in gray. The position of the substitution of tomato SGR1 protein in *gf* mutant in amino acid 143 (Arg143→Ser143) is indicated by *star*.

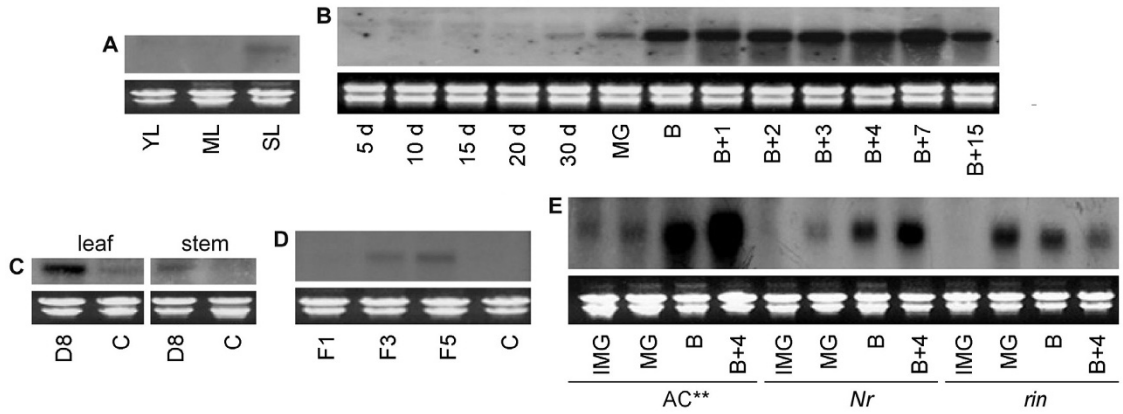


Fig. 3. Expression of *LeSGR1* gene analyzed by Northern hybridization. *A* - expression in young leaves (YL), mature leaves (ML) and senescent leaves (SL) of wild type tomato. *B* - Expression in wild type fruits in 5, 10, 15, 20 and 30 d post anthesis and in stages of mature green (MG), breaker (B) and post-breaker stages (B+1, B+2, B+3, B+4, B+7 and B+15 denoting 1, 2, 3, 4, 7 and 15 d after breaker. *C* - Expression in wild type leaves and stems after 8 h of water stress treatment in comparison with the control (C). *D* - Expression in wild type tomato leaves after flooding of plants for 1, 3 and 5 d in comparison with the control (C). *E* - Expression in fruits of 4 stages as follows: in about 20 d after anthesis when the fruits were immature green (IMG), MG, B, and B+4 in wild type ($AC^{++}$), ethylene-insensitive mutant (*Nr*), and ripening inhibitor (*rin*) transgenic tomatoes. The amount of total RNA sample for Northern blot was 20 μg, which was quantified with UV spectrophotometry.

serine 205 and serine 217) in the deduced protein are predicted with *NetPhos 2.0* server (Blom *et al.* 1999). No transmembrane helices exist according to the prediction by *TMHMM* v. *2.0* server. The protein probably locates in chloroplasts and distributes in stroma and on the stroma side of thylakoid membrane, as predicted with *WoLF PSORT* package (http://wolfpsort.seq.cbrc.jp/).

Comparison of the nucleotide sequence of *SGR1* cloned from the wild type with that from *gf*/*gf* mutant tomato indicated that there is a single nucleotide substitution of T429 for A429 in coding domain sequence (CDS) in the *gf*/*gf* mutant. This nucleotide substitution leads to a single amino acid change from Arg143 to Ser143 (Fig. 2).

**Expression pattern of *LeSGR1* gene:** Northern blot hybridization indicated that the signal intensity of the hybridization band for *LeSGR1* depended on the stage of development of the fruit or leaf investigated. *LeSGR1* mRNA was present mainly at senescent leaves, but almost undetectable in young leaves and mature leaves (Fig. 3*A*). In tomato fruits (Fig. 3*B*), the expression of *LeSGR1* was barely detected within the first 20 d after anthesis, but began to increase from the 30$^{th}$ day after anthesis, reached a very high level at breaker (B) stage and kept at this high level from B to B+7 stage. At B+15 stage, the transcriptional level of *LeSGR1* showed some decrease. This is probably because cell apoptosis began after the fruit was ripe. These results suggested that the expression of *LeSGR1* was induced and regulated by the signals of fruit ripening and leaf senescence. After exposure to 8-h water deficit, *LeSGR1* was amply expressed in tomato leaves and stems, much higher than in the control (Fig. 3*C*). The expression level of *LeSGR1* in leaves was obviously higher than that in stems. Under flooding, the *LeSGR1* mRNA in leaf was barely detected after 1 d of treatment, while the *LeSGR1* expression level increased rapidly with the flooding prolongation (Fig. 3*D*). Under water-deficit and flooding, the expression levels of *LeSGR1* were enhanced. We speculated that these stresses might promote senescence of leaves (Hurng *et al.* 1994, Pic *et al.* 2002), and subsequently the senescence regulated tissue-specific expression of *LeSGR1*.

In order to reveal whether *LeSGR1* was related with ethylene signal transduction pathway, we examined the expression of *LeSGR1* at different stages of fruit development in wild type tomatoes ($AC^{++}$), ethylene-insensitive mutant (*Nr*) and ripening inhibitor (*rin*) tomatoes (Fig. 3*E*). The LeSGR1 mRNA was not detectable in immature green (IMG) fruits of either *Nr* or

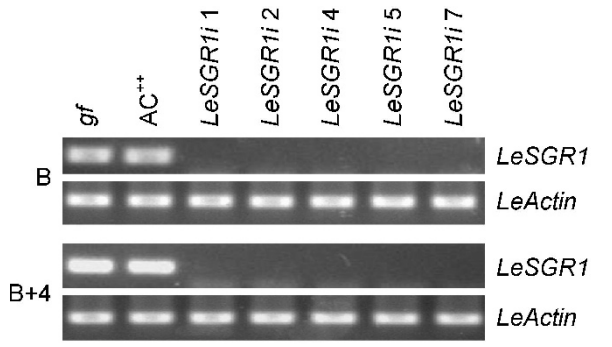


Fig. 4. Silencing effect of *LeSGR1* gene in the transgenic plants. Semiquantitative RT-PCR analysis of LeSGR1 in fruits of $AC^{++}$, *gf* mutant and five *LeSGR1* dsRNA-mediated genetic interference lines at the B (breaker stage) and B+4 (4 d after breaker) development stages. Tomato *actin* gene (*LeActin*) was used as internal control. Primers for PCR were as follows. *LeSGR1*, forward: 5'-AACTCCCTGTGGTTCTCAAG-3', reverse: 5'-GGAAAGCAACAGGCACAAGC-3'; *actin*, forward: 5'-TGAAATGTGACGTGGATATTAGG-3', reverse: 5'-TGAGGGAAGCCAAGATAGAGC-3'.

*rin* mutant tomato plants, but was noticeably more in MG fruits of *rin* plants than *Nr* plants. The expression of this gene in *Nr* fruits increased gradually from MG to B+4, while, decreased slowly in *rin* fruits. The *LeSGR1* level in wild type tomato was much higher than that in *Nr* and *rin* mutants at the four stages of fruit development, IMG, MG, B and B+4, suggesting that *LeSGR1* expression is up-regulated by ethylene.

**LeSGR1 dsRNA-mediated genetic interference mutant exhibits a stay-green phenotype:** To further probe into the functions of *LeSGR1*, we constructed an RNAi expression vector targeting *LeSGR1* gene and transformed it into $AC^{++}$ tomato via *Agrobacterium tumefaciens*-mediated transformation. We obtained 23 transgenic explants, selecting by kanamycin resistance, and denominated as *LeSGR1i*. PCR analysis with the marker gene of neomycin phosphotransferase II (npt II) indicated that 19 of those transgenic plants contained specific fragment of npt II (data not shown). In order to determine the expression level of *LeSGR1* in transgenic plants, semiquantitative RT-PCR analysis was performed. The total RNAs, extracted from transgenic tomato fruits at B and B+4 development stages, were used as template. The expression of *LeSGR1* mRNA was suppressed to a great extent in most of transgenic plants. Fig. 4 shows the interference effects on expression of *LeSGR1* in five transgenic tomato plants, *LeSGR1i* 1, *LeSGR1i* 2, *LeSGR1i* 4, *LeSGR1i* 5 and *LeSGR1i* 7. Compared to the wild type ($AC^{++}$) and *gf* mutant tomatoes, no obvious *LeSGR1* transcript has been detected in B and B+4 fruits of transgenic plants. The ripe fruits of the *LeSGR1i* plants displayed a rusty red color (Fig. 5*A*). Furthermore, mature leaves of transgenic plants had deeper green color than that of $AC^{++}$ (Fig. 5*B*), and leaves on the lowermost nodes did not lose their chlorophyll. The very old leaves eventually withered and abscised without any visible loss of chlorophyll (Fig. 5*B*). Leaves of *LeSGR1i* plants also retained chlorophyll when detached and kept in the dark (Fig. 5*C*). These results indicated that *LeSGR1i* plants exhibited stay-green phenotype that was observed in the *gf* mutant (Akhtar *et al.* 1999).

**LeSGR1 silence inhibits chlorophyll degradation:** Quantitative determination of chlorophyll contents supported the visual observations. A decline of only 13.1 % in chlorophyll was found in ageing *gf* leaves, as compared with 64.7 % loss of chlorophyll in comparable wild type leaves. The speed of chlorophyll degradation in the ageing leaves of five transgenic lines was close to that in *gf* mutant (Fig. 6*A*). In fruit, almost all chlorophyll was lost from the wild type 4 d after the start of colour change (B+4). But at a comparable stage (B+4) 70.2 % of chlorophyll was retained in *gf* mutant, 60.1 % in *LeSGR1i* 1, 63.9 % in *LeSGR1i* 2, 65.4 % in *LeSGR1i* 4, 56.3 % in *LeSGR1i* 5, 71.5 % in *LeSGR1i* 7 (Fig. 6*B*).

All these results suggested that *LeSGR1* gene silencing inhibited chlorophyll degradation and exhibited a stay-green phenomenon, which was similar to that in the *gf*

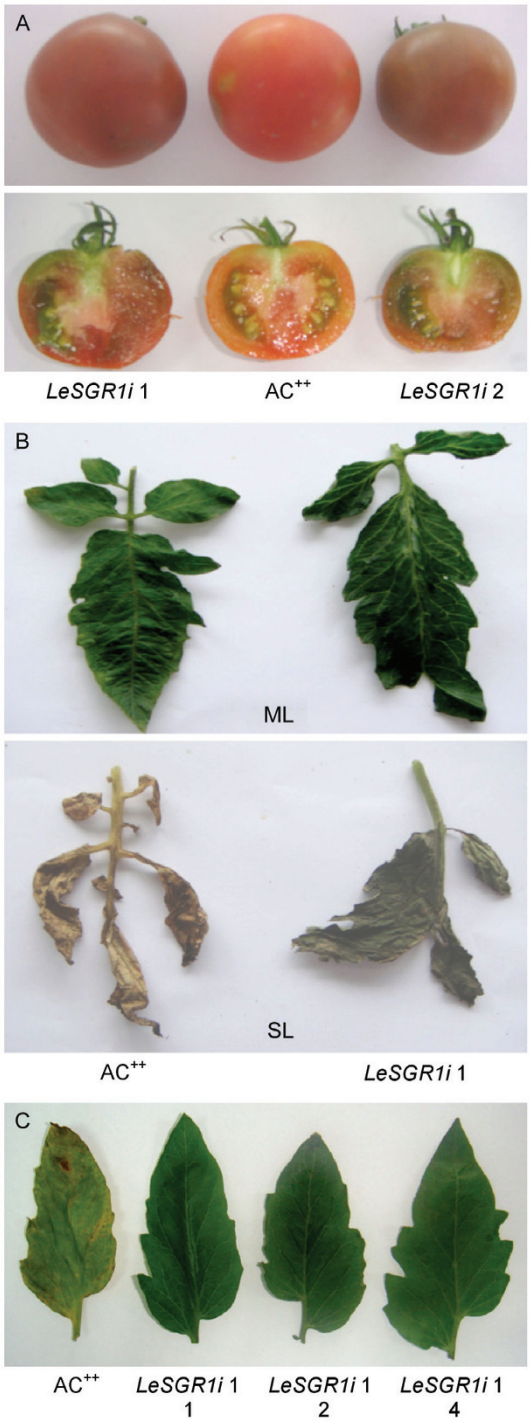


Fig. 5. Phenotype analysis of transgenic plants. *A* - Phenotype of ripe wild-type and transgenic fruits at B+4 stage which is similar to tomato *gf* mutant. *B* - Phenotype of mature (ML) and senescent (SL) leaves of transgenic plant. Mature leaves of transgenic plants had deeper green colour than that of wild type ($AC^{++}$). The very old leaves in transgenic plants eventually withered and abscised without any visible loss of chlorophyll. *C* - Dark-induced senescence of leaves of wild type and transgenic plants. After 7 d, the wild type leaves turned yellow, but transgenic tomato plants leaves remained green.

mutant, where a homolog of *LeSGR1* was identified to have a single nucleotide change that led to an amino acid substitution. Thus, we conclude that *LeSGR1* gene is crucial to chlorophyll degradation and the mutation of SGR protein is responsible for the *gf* tomato mutant properties.

## Discussion

Leaf senescence is a programmed process of efficient nourishment reallocation regulated by some nuclear genes (Yoshida 1961). Kawakani and Watanabe (1993) discovered that senescence-associated protein encoded by the nuclear gene was effectively transported to chloroplasts in stay-green mutant (Bf993) and wild type (Rossa) of fescue. However, the correlation between the senescence-associated protein and chloroplast senescence has not been proven unequivocally. Very recently, research on the rice *sgr* mutant revealed that SGR protein is a chloroplast protein and *SGR* mRNA transcription is highly induced by the onset of senescence (Park *et al.* 2007). Similarly, the LeSGR1 gene is almost undetectable in the immature tissues of tomato plants, and is highly induced by senescence signal. This suggests that LeSGR1 protein is a senescence-associated protein. More interestingly, drought and flooding, which are unfavorable factors to growth, increase the expression of the gene. The expression level of *LeSGR1* in wild type tomato is much higher than that in both ethylene-insensitive mutant (*Nr*) and ripening inhibitor mutant (*rin*), implying the regulation by ethylene and ripening factors. It is well known that ethylene is a gaseous plant hormone that plays a key role in leaf senescence, fruit ripening, abscission and responses to abiotic and biotic stresses.

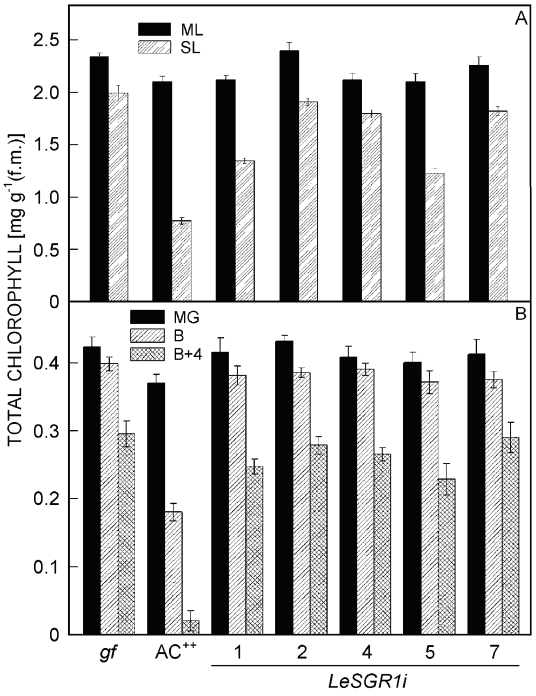


Fig. 6. *A* - Total chlorophyll content in mature and senescent leaves of $AC^{++}$, *gf* mutant and five *LeSGR1* dsRNA-mediated genetic interference lines. *Black bar* mature leaf (ML), *gray bar* senescent leaf (SL). *B* - Total chlorophyll content in MG, B and B+4 fruits of $AC^{++}$, *gf* mutant and five LeSGR1 dsRNA-mediated genetic interference lines. *Gray bar* MG fruit, *black bar* B fruit, *light gray bar* B+4 fruit. *Error bars* represent the standard deviations of three independent experiments.

In the nucleotide sequence of *LeSGR1*, there is a single nucleotide substitution of T429 in the conservative coding domain sequence (CDS) in the *gf* mutant for A429 in the wild-type. This nucleotide substitution resulted in a single amino acid change from Arg143 to Ser143. Jiang *et al.* (2007) reported that there was a single nucleotide substitution of A251 to G251 in the stay-green rice mutant coding domain sequence (CDS) that produced a single amino acid change from Tyr84 to Cys84. The transgenic tomato plants from RNA interference (RNAi), with an inhibited expression of *LeSGR1* gene, exhibit a stay-green phenotype, indicating that the inhibition of Chl degradation in tomato is due to the absence of LeSGR1 protein. Based on these findings, we conclude that the antisense mutation of LeSGR1 protein might be responsible for the stay-green phenotype of *gf* mutation.

Although we have proved that LeSGR1 protein plays an important role in Chl degradation, its specific function is still obscure. It was reported previously that Chl degradation is a multistep pathway catalyzed by numerous enzymes. Lack of any of them can influence the Chl catabolism and result in stay-green phenomenon (Li *et al.* 2010). However, stay-green phenotype of *gf* mutant appear not to be caused by this reason, because the activities of all the enzymes of Chl catabolic pathway were not affected in *gf* mutant (Akhtar *et al.* 1999). Very recently, the SGR protein in rice has been shown to have ability to bind light harvesting chlorophyll-binding protein (LHCP) 2 in *sgr* mutant (Park *et al.* 2007). If the dissociation of Chls from the Chl-protein complexes is a prerequisite for the degradation of Chls, this finding suggests that SGR protein may be related to LHCP 2 destabilization. Despite the authenticity of this assumption, the way in which SGR protein leads to destabilization of LHCP 2 is not yet clear. More evidences are necessary to support this assumption. In addition, previous research revealed that the antisense mutation in rice *sgr* mutant did not lose the capacity to bind LHCP 2 *in vivo*. How does the mutation affect the function of SGR protein is still unclear. Thus, further biochemical or relevant molecular study between SGR protein and LHCP 2 is necessary to elucidate the event of LHCP 2 disassembly.

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