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Chlorophyll breakdown during pepper fruit ripening in the *chlorophyll retainer* mutation is impaired at the homolog of the senescence-inducible stay-green gene

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Abstract The pepper *chlorophyll retainer* (*cl*) mutation is characterized by inhibition of chlorophyll degradation during fruit ripening. Ripe fruit of *cl* pepper containing chlorophyll and red carotenoids is brown, while ripe fruit containing chlorophyll and yellow carotenoids is green. In addition to the inhibitory effect during fruit ripening caused by *cl*, we show that chlorophyll degradation is inhibited during natural and dark-induced leaf senescence. Therefore, the *cl* mutation has the characteristics of the *stay-green* (*sgr*) mutants described in many other species. Upon the recent discovery of the *SGR* gene in various plant species, we isolated pepper *SGR* (*CaSGR*) and found that it genetically cosegregates with *cl* in a BC1 mapping population. Furthermore, sequencing the wild-type and mutant alleles revealed an amino-acid substitution of tryptophan (aromatic amino acid) to arginine (basic amino acid) at position 114 in the protein sequence. The single-nucleotide polymorphism (SNP) that differentiates the wild-type and mutant alleles was exploited to develop a PCR marker useful for marker-assisted selection. Expression of *CaSGR* as measured by semiquantitative RT-PCR was mostly induced upon fruit ripening and to a lesser extent upon leaf senescence. Taking together, our genetic, sequence and expression data all indicate that *CaSGR* is a candidate for controlling the *cl* mutation in pepper.

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Introduction

Chlorophyll is the principle molecule absorbing sunlight in the process of photosynthesis and it gives plants their green color. The basic structure of chlorophyll is a porphyrin ring bonded to a central magnesium ion and to a phytol side chain. During leaf senescence and fruit ripening, the green color is degraded due to the breakdown of chlorophyll to colorless products. The early steps of the chlorophyll catabolic pathway consist of four enzymatic activities (Hörtensteiner 2006). In the first step, the phytol chain is cleaved from chlorophyll by chlorophyllase to produce chlorophyllide. Mg ion of chlorophyllide is removed by Mg-dechelatase to produce pheophorbide *a*. The porphyrin ring of the green pheophorbide *a* is cleaved by pheophorbide *a* oxygenase (PaO), resulting in a red chlorophyll catabolite which is subsequently converted to primary fluorescent chlorophyll catabolite (pFCC) by red chlorophyll catabolite reductase (RCCR). pFCC is further metabolized by less characterized steps to nonfluorescent chlorophyll catabolites. Unlike leaf senescence, chlorophyll degradation at fruit ripening is accompanied by the conversion of chloroplasts to chromoplasts; nevertheless, the enzymatic activities responsible for chlorophyll breakdown are the same in both tissues (Moser and Matile 1997).

Mutants, often called *stay green* (*sgr*), in which chlorophyll catabolism during leaf senescence is inhibited, have been described in a number of species, including *Festuca*, pea, soybean, rice and *Arabidopsis* (Bachmann et al. 1994; Cha et al. 2002; Luquez and Guiamet 2002; Armstead et al. 2006, 2007; Ren et al. 2007). In addition to these leaf *stay-green* mutants, fruit *stay-green* mutants controlled by single-recessive genes have been reported in tomato (*green flesh*, *gf*) and pepper (*chlorophyll retainer*, *cl*) (Akhtar et al. 1999; Efrati et al. 2005). The color of ripe

cl fruit may be brown or green, depending on the genotype at the *Y* locus controlling the accumulation of red carotenoids. In plants having a dominant allele at *Y*, the fruit is brown because of simultaneous accumulation of chlorophyll and red carotenoids. In plants homozygous for the recessive *y* allele, the fruit is green because the red pigments are not produced.

The pepper *cl* locus was recently mapped to chromosome 1 (Efrati et al. 2005). Candidate genes from the chlorophyll-catabolism pathway, including chlorophyllase and PaO, did not map to the corresponding genomic loci in tomato, indicating that genes other than those coding for enzymes in the pathway may control the mutation. Biochemical analyses of brown-fruited peppers showing characteristics of *stay-green* mutants indicated a possible lesion in the PaO activity as the cause of the stay-green phenotype because unlike in wild-type fruits, chlorophyllide and pheophorbide *a* are accumulated in the ripe mutant fruits (Roca and Minguez-Mosquera 2006). PaO is a key enzyme controlling chlorophyll breakdown because it is activated during senescence and ripening and is responsible for loss of green color (Moser and Matile 1997; Pruzinska et al. 2003). The accumulation of pheophorbide *a* or a low level of the PaO activity in *stay-green* mutants of other plant species also point to a lesion in the ring-opening step as the cause for the mutant phenotype (Vicentini et al. 1995; Thomas et al. 1996; Pruzinska et al. 2003; Ren et al. 2007).

Recently, *SGR* gene homologs were isolated from *Festuca*, pea, rice and *Arabidopsis* and shown to control stay-green phenotypes in senescent cotyledons and leaves (Armstead et al. 2006, 2007; Park et al. 2007; Ren et al. 2007; Sato et al. 2007). The protein contains a chloroplast transit peptide and gene expression is induced during senescence (Hörtensteiner 2006; Armstead et al. 2007; Park et al. 2007). Subcellular localization using an SGR:GFP fusion in rice indicated that SGR is a chloroplast protein (Park et al. 2007). The SGR protein was also found in the proteome of pepper chromoplast (Asim Siddique et al. 2006). Although the function of the gene has not yet been proved, it is postulated to modulate the activity of PaO (Armstead et al. 2006; Ren et al. 2007). In rice, SGR was shown to have a role in the disassembly of the light-harvesting chlorophyll–binding protein (LHCP) complexes, a prerequisite for chlorophyll degradation: the disassembly fails in the *sgr* mutant (Park et al. 2007). SGR was postulated to be a key regulator of the disassembly process through direct interaction with the protein complex.

The conservation of *SGR* function across diverse dicot and monocot species and the similar biochemical properties of pepper *cl* and *stay-green* mutants led us to test *sgr* as a candidate for controlling the pepper mutation. In this paper, we describe genetic and molecular analyses that provide evidence that pepper *SGR* is the gene responsible for the *chlorophyll retainer* phenotype.

Materials and methods

Plant material

The mapping population of *cl* is described by Efrati et al. (2005). Briefly, it consists of 198 BC1 progenies from an inter-specific cross of *Capsicum annuum* inbred line 4590 (green ripe fruit) and *C. chinense* PI 159234 (red ripe fruit). The F1 was crossed to 4590 to create the BC1 population. The two parents differ at two loci controlling fruit color. PI 159234 is homozygous for the dominant alleles at *CL* and the *Y* locus controlling red pigment accumulation in the ripe fruit. Line 4590 is a double recessive mutant at both loci. BC1 progeny were scored visually for ripe fruit color: brown and green fruits were scored as mutants at the *cl* locus and red and yellow fruits were scored as wild-type genotypes (Fig. 1). Because isogenic plant material to the *cl* mutation was not available, we used Maor, a *C. annuum* wild-type blocky-fruited inbred for phenotypic comparisons.

Gene isolation

Pepper EST KS17053G06 containing the partial sequence of *SGR* was used for initial RT-PCR amplification of the gene using total RNA of Maor. The RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) and was reverse-transcribed with a *REVERSE*-IT™ 1st strand synthesis kit (ABGene). The primers used were 5′-TCTCTTTCACACACACACAGT-3′ (forward) and 5′-GCAACCACTTCATCCCTTTG-3′ (reverse). The EST contains 426 bp of the 5′ end of the open reading

Fig. 1 BC1 progeny derived from a cross of F1 (red × green) × green. The red-fruited parent (PI 159234) is homozygous for the dominant alleles at the *Y* and *CL* loci, while the green-fruited parent (4590) is a double recessive mutant (*yy/clcl*). Four colors of fruits were obtained from the segregation of the *Y* and *CL* loci. Red and yellow fruits have the dominant allele *CL*, while brown and green fruits are homozygous recessive *clcl*

frame (ORF) plus 302 bp of the 5′ untranslated region. To isolate full-length cDNA, we employed 3′-RACE using RNA extracted from ripe fruit of both wild-type and mutant parents. A tailed oligo-(dT) primer (5′-GTTTTCCCAGTC ACGACGTTTTTTTTTTTTTTT-3′) was used for single-strand cDNA synthesis (Frydman et al. 2004). For RT-PCR, we used the gene-specific primer 5′-TCTCTTTCA CACACACACAGT-3′ with a tail-primer (5′-GTTTTCCC AGTCACGACG-3′). Cloned PCR products were sequenced by Hy Laboratory LTD, Israel.

RFLP analysis and development of dCAPS marker

Restriction fragment length polymorphism (RFLP) analysis with parental DNA and segregating progenies using the full-length cDNA of pepper *SGR* was performed as described by Rao et al. (2003). To generate the dCAPS marker (Michaels and Amasino 1998), we used a 25-bp forward primer 5′-TCAAAGGGATGAAGTGGTTGC AGGA-3′ from exon 1 and a reverse primer 5′-AGGA ACACGGCCGAACGATAT-3′ from intron 1 (intron sequence was derived from PCR amplification of genomic DNA using the original primers from the EST). The introduced "G" instead of "A" at position 24 in the forward primer created a mismatch that generated a *Fok*I restriction site in the amplification product using wild-type but not mutant DNA. Segregation of the dCAPS marker was followed in an F2 population (100 individuals) from a cross between 4590 and a wild-type *C. annuum* line 1154.

Expression analysis

To determine the expression pattern of *CaSGR*, RNA was extracted from wild-type and mutant leaves and fruits and treated with DNase to eliminate genomic DNA. Leaves were harvested at three developmental stages, such as young, fully expanded and senescent. Similarly, fruits were harvested as young, mature green and ripe. Semi-quantitative RT-PCR was performed using forward primer 5′-TCTCTTTCACACACACACAGT-3′ and reverse primer 5′-GGCACAACCCAACTTTACAA-3′. Ubiquitin (SGN-U198046) was used as a reference for determining relative expression level using forward primer 5′-AATAAGGATGCAGGCTTCAAGGGC-3′ and reverse primer 5′-TGATGTCACGGGACCGAAGA-AGAT-3′. Linear amplification was performed by using 20 cycles of PCR. The amplification products were run on an agarose gel, blotted onto a nylon membrane (Amersham N^+) and hybridized with the respective probes. After exposing the filters to phosphorimager Fuji NFL 5000, the hybridization signals were quantified using the Image Gauge software.

The sequence of the pepper *SGR* (*CaSGR*) cDNA was deposited in GenBank (accession number EU196733).

Results

Phenotypic effect of *cl* during leaf senescence

The *cl* mutation was previously characterized for its inhibitory effect on chlorophyll degradation at fruit ripening (Efrati et al. 2005; Roca and Minguez-Mosquera 2006). A fivefold increase in chlorophyll content was observed in ripened fruit of the *cl* line 4590 when compared to wild-type fruit (Efrati et al. 2005). However, no data were available on whether the mutation also affects chlorophyll degradation during leaf senescence. We therefore followed leaf appearance under normal growing conditions in the greenhouse and after induction of senescence. Two weeks after germination, the cotyledons of the wild-type parent Maor became yellow. While some yellowing was observed for the cotyledons of 4590, overall they remained greener than those of Maor. No further difference was observed in the appearance of these genotypes under normal growing conditions up to fruit ripening. However, a difference in appearance was observed when the plants were deprived of fertilization: Maor leaves became yellow retaining only 20% of the initial chlorophyll, while those of 4590 also turned yellow but overall remained greener than those of Maor, retaining 37% of the initial chlorophyll (Fig. 2). Furthermore, we incubated fully expanded detached leaves in the dark and measured the reduction in chlorophyll content after 14 days (Fig. 2). While in leaves of Maor there was a 41% reduction in chlorophyll content (from 0.89 ± 0.15 to 0.53 ± 0.09 mg/g) resulting in partial yellowing, in leaves of 4590 we measured a 23% reduction in chlorophyll content (from 0.82 ± 0.08 to 0.63 ± 0.1 mg/g) with no

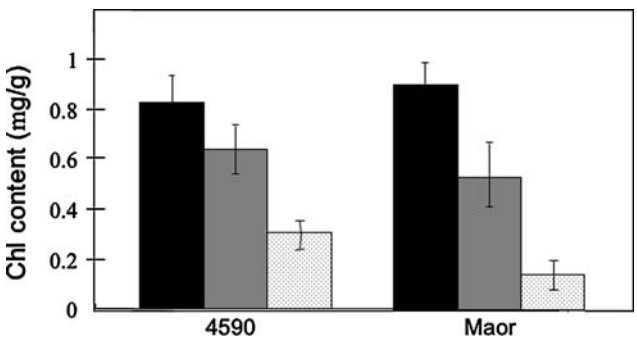

Fig. 2 Chlorophyll content in leaves of 4590 and Maor before and after induction of senescence. *Black bar* leaf under normal growing conditions. *Gray bar* detached leaf after 2 weeks in the dark. *Dotted bar* leaf 4 weeks after deprivation of fertilization under natural light conditions. *Error bars* represent standard errors derived from five replications

significant yellowing. These experiments revealed that pepper *cl* has the characteristics of a leafy *stay-green* mutant, although inhibition of chlorophyll degradation during leaf senescence is less dramatic than that in *stay-green* mutants of other plant species.

Gene isolation and sequence analysis of wild-type and mutant alleles

The full-length cDNA of *CaSGR* consists of 1257 bp with a 798-bp ORF that codes for a protein of 266 amino acids. Comparison of the sequence of wild-type and mutant alleles revealed a single-nucleotide change from T in the wild-type allele to C in the mutant allele at nucleotide 340 of the ORF. This single-nucleotide polymorphism (SNP) led to an amino-acid substitution of tryptophan (aromatic amino acid) to arginine (basic amino acid) at position 114 in the protein sequence (Fig. 3). Searching the NCBI nucleotide database by BLAST revealed sequence similarity to SGR sequences from multiple plants such as tomato, tobacco, Arabidopsis, pea and rice but no homology to other recently isolated stay-green genes (Kusaba et al. 2007).

Cosegregation of *CaSGR* and *cl* and development of dCAPS marker

To test the linkage relationship between *CaSGR* and *cl*, we performed RFLP analysis using the parents of the mapping population. A survey of parental DNA digested with eight restriction enzymes revealed polymorphism with the enzymes *Hind*III and *Xba*I. Southern analysis detected two bands with similar intensities, indicating the possible occurrence of a second *SGR* homolog in the pepper genome (Fig. 4a). Only one of the two bands was polymorphic and its segregation was followed in the mapping population. Linkage analysis of *cl* and *CaSGR* revealed complete cosegregation of the two loci. To utilize the SNP that differentiates the mutant allele from the wild type for marker-assisted selection, we developed a codominant dCAPS marker by introducing a *Fok*I restriction site into the amplification product of the wild-type DNA (Fig. 4b). Scoring the dCAPS marker in a new F2 population from the cross of 4590 and a wild-type parent red-fruited *C. annuum* 1154 revealed complete linkage with the segregating phenotypes.

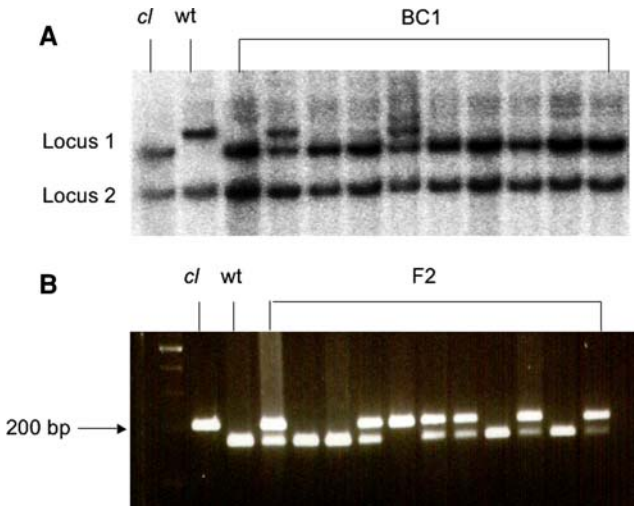

Fig. 4 RFLP and dCAPS analyses of *CaSGR*. **a** RFLP analysis of *CaSGR* using genomic DNA of the parents and BC1 population digested with *Xba*I. **b** dCAPS analysis of *CaSGR* in the parents and F2 population. The PCR product was digested after amplification by *Fok*I. *cl* and wt parents have green ripe and red fruits, respectively

Expression of *CaSGR* during leaf and fruit development

To determine the expression pattern of *CaSGR* during leaf and fruit development, we performed semiquantitative RT-PCR analyses on RNA extracted from leaves and fruits from different developmental stages. A low level of *CaSGR* expression was detected in young and fully mature leaves as well as in young and mature green fruit (Fig. 5). Expression was induced in senescent leaves but the most marked expression was detected in ripe fruit. The expression level of *CaSGR* in ripe fruit was significantly lower in the mutant parent compared to the wild type.

Discussion

Stay-green phenotypes, mostly associated with leaf senescence, are known in many plant species (Thomas and Smart 1993). For fruit-bearing crops, only a few stay-green phenotypes have been described. In banana peel, chlorophyll catabolism is inhibited at temperatures higher than 24°C; however, this stay-green phenotype is not associated with inhibition of PaO as in the case of *stay-green* mutants in other plants (Drury et al. 1999). Tomato *gf*, in which chlorophyll is retained in the ripe fruit, is also characterized by

Fig. 3 Deduced amino-acid sequence of *CaSGR*. The position of the substitution in amino acid 114 (W114R) is *underlined*

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MGTLTASLVAPSKLNPEKHSSLFVYKTRRKSHKNQSIVPVARLFGPAIFEASKLKVLFLG 60
VDEKKHPGKLPRTYTLTHSDITSKLTLAISQTINNSQLQGWYNRLQRDEVVAEWKKVKGK 120
MSLHVHCHISGGHFMLDLFARLRYYIFCKELPVVLKAFVHGDENLLKNYPELQQALVWVY 180
FHSNIQEFNKVECWGPLKDAASPSSSGVGGGMNTSFTSNSNIKWNLPKPCEETCTCCFPP 240
MSVIPWPSTTNVENGTIQQGLQEQQS 266
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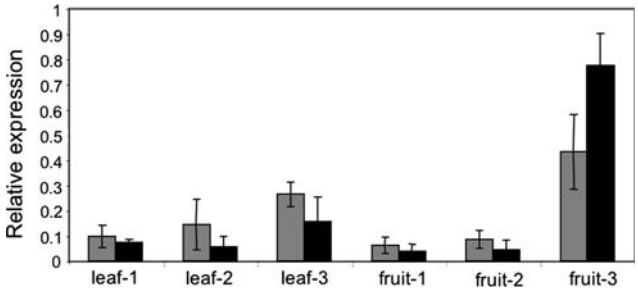

Fig. 5 Semiquantitative RT-PCR analysis of *CaSGR*. RNA was extracted from young leaf (*leaf-1*), fully expanded leaf (*leaf-2*), senescent leaf (*leaf-3*), young green fruit (*fruit-1*), mature green fruit (*fruit-2*) and ripe red fruit (*fruit-3*). PCR products were blotted on agarose gels and hybridized with ^{32}P-labeled *CaSGR* and ubiquitin probes. *Black bar* Maor, *gray bar* 4590

a leaf stay-green phenotype (Akhtar et al. 1999). Similarly, the stay-green phenotype in pepper *cl* which was originally associated with a fruit-specific phenotype is shown here to be associated with inhibition of chlorophyll degradation in senescent leaves as well. Pepper *cl* and tomato *gf* may be orthologous loci because they have similar phenotypes and are assigned to the same chromosomes. The genomic region containing *cl* in chromosome 1 in pepper corresponds to chromosome 8 in tomato, to which *gf* is assigned (Kerr 1957). However, the molecular basis of *gf* and its precise map position are not yet known.

The fruit of the *cl* mutant line 4590 remains green at ripening: only at the over-ripe stage does some yellowing appear. Therefore, the chlorophyll-catabolism pathway in ripe fruit is not completely inhibited but rather, greatly delayed. This behavior is similar to the phenotype of the brown-fruited pepper cultivar Negral in which chlorophyll catabolism is completely inhibited during the first 60 days after fruit set and partially inhibited at 75 days after fruit set (Roca and Minguez-Mosquera 2006). The partial inhibition of chlorophyll degradation in line 4590, as manifested by the appearance of yellow color in the late stages of ripening as well as partial yellowing during leaf senescence, could be regarded as a weak phenotype of the *cl* mutation resulting from an amino-acid substitution in the protein.

The chlorophyll-catabolism pathway in pepper fruit has been shown to be similar to that operating in senescent leaves (Moser and Matile 1997) and therefore, the genetic control of fruit stay-green phenotypes is expected to be similar to other known leaf phenotypes. The accumulation of the chlorophyll catabolites chlorophyllide *a* and pheophorbide *a* during ripening of Negral fruit is indicative of a reduction in PaO activity as the cause of the stay-green phenotype, similar to the postulated mechanism in leaf *stay-green* mutants. The isolation of *SGR* homologs in several plant species has enabled testing of the hypothesis that the genetic control of *stay-green* in ripe fruit is similar to that in senescent leaves. Our genetic analysis showing complete cosegregation of *CaSGR* with *cl*, together with the change in a single amino acid of SGR in the mutant, strongly support the conclusion that *CaSGR* corresponds to the *cl* locus. Our results indicate that SGR has a conserved role in chlorophyll catabolism not only during leaf senescence, but also during fruit ripening. Similar to the *cl* mutation, a single base change causing a missense mutation was identified as the cause of mutation in a rice *sgr* mutant (Park et al. 2007). In addition to the missense mutation observed in *cl*, reduced *CaSGR* expression was found in the ripe fruit of the mutant compared to wild-type fruit. This is in contrast to the non-significant higher expression level of *CaSGR* in leaves and immature fruit in *cl* compared to Maor. The differences in expression level in both genotypes, may not be associated with the presence or absence of the mutation, but rather to differences in the genetic background, as induction of expression at fruit ripening and to a lesser extent at leaf senescence is observed in both genotypes.

Our results indicate that *CaSGR* is expressed at low levels in the leaves of wild-type and mutant lines. We did not observe the strong induction in expression that is commonly found in senescent leaves in other plant species. Because SGR is conserved across many plant species and its function is likely to be required for normal leaf senescence in pepper, we postulate that there may be a second *SGR* gene in pepper that functions at leaf senescence. This hypothesis is supported by the Southern analysis in which a second non-segregating band was detected in several restriction digests of the parental DNA. In *Arabidopsis*, two SGR homologs with 70% amino-acid identity exist, both showing similar patterns of expression (Park et al. 2007). Similarly, in soybean, two SGR homologs exist with 92% amino-acid identity. In pepper, as well as in tomato, a single EST was found in a cDNA library of ripe fruit; EST data from libraries of senescent leaves are not available. It is therefore possible that an additional diverged gene with redundant function exists.

The *cl* mutation controlling brown mature fruit color was initially described by Smith (1948). The use of brown fruits has spread worldwide but it is not known whether the mutation found in line 4590 is responsible for the phenotype in other accessions. We tested two *C. annuum* lines that putatively have the *cl* mutation: a breeding line, 1133, with green mature fruit obtained from Dr. C. Shifriss at The Volcani Center and a USDA accession with brown fruit (PI 439289). Both lines shared the mutation found in line 4590 as revealed by sequencing and dCAPS analysis. A more comprehensive survey is required to determine whether additional genes or alleles control the stay-green phenotype in other *Capsicum* accessions and species.

Reduced activity of PaO and accumulation of pheophorbide *a* in several *sgr* mutants has led to the hypothesis that

SGR is a modulator of PaO (Armstead et al. 2006). PaO may be part of a protein complex that mediates the degradation of the thylakoid membranes. The recent finding that rice SGR is necessary for the degradation of the thylakoid membranes' LHCP has led to an alternative hypothesis in which SGR-mediated degradation of the complex gives chlorophyll access to the catabolic enzymes (Park et al. 2007). LHCPII retention in several *sgr* mutants during leaf senescence or fruit ripening (Cheung et al. 1993; Akhtar et al. 1999; Pruzinska et al. 2003) supports the involvement of SGR in the process of protein destabilization. Interestingly, a second, *stay-green* mutant in rice that is nonallelic to *sgr*, *non-yellow coloring 1*, was recently shown to be required for the degradation of LHCPII (Kusaba et al. 2007). Therefore, it appears that different genes mediate degradation of the LHCP complex and interference with their action is a key feature in stay-green phenotypes.

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