Supplemental Data

Regulation of carotenoid and chlorophyll pools in hesperidia, anatomically unique fruits found only in *Citrus*

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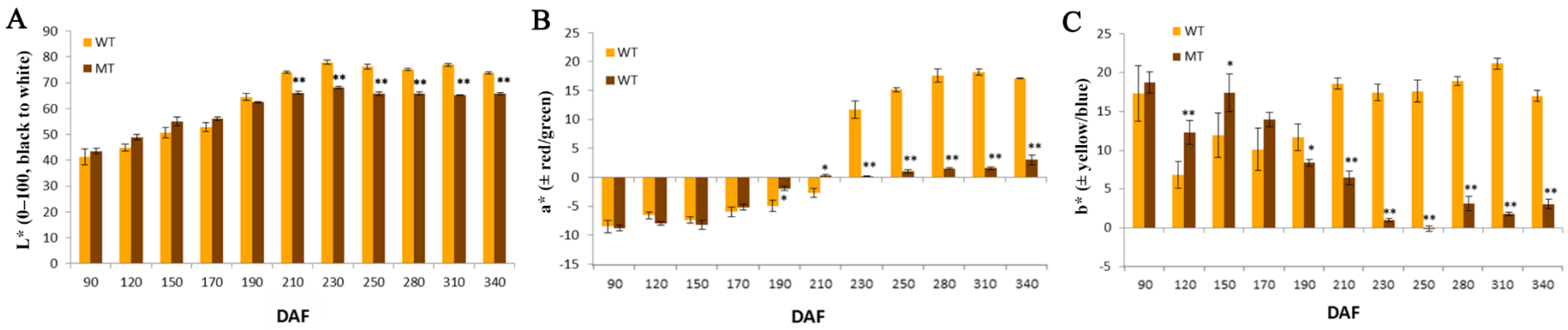
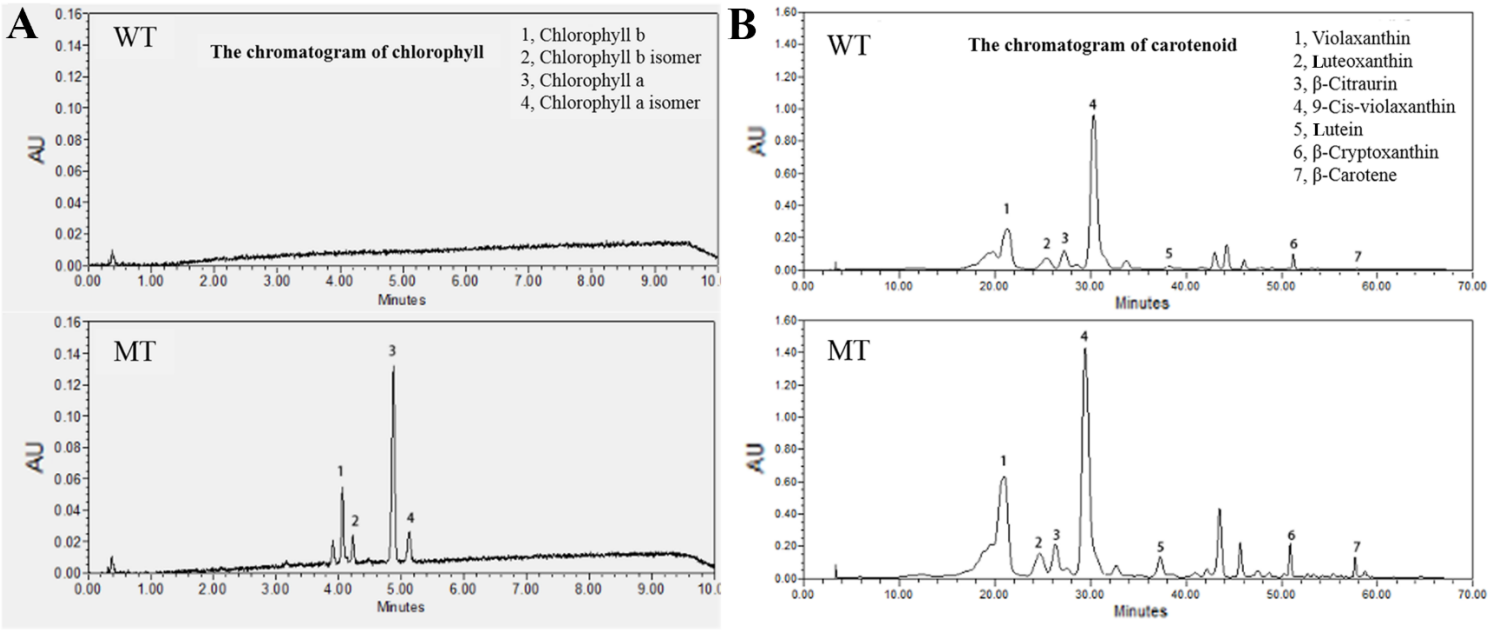
Supplemental Figure S1



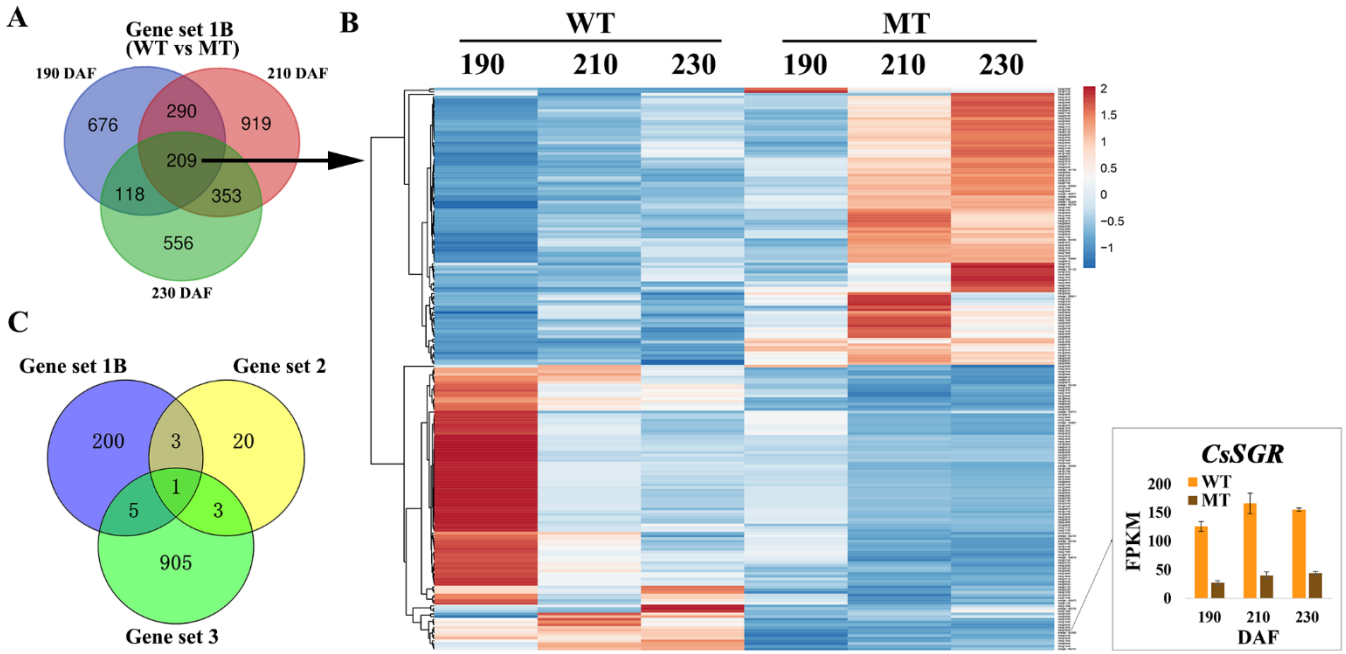
Supplemental Figure S1 Assessment of color values (L*, a* and b*) in developing fruit of WT and MT at various days after flowering (DAF). (A) L* values. (B) a* values. (C) b* values.

Supplemental Figure S2



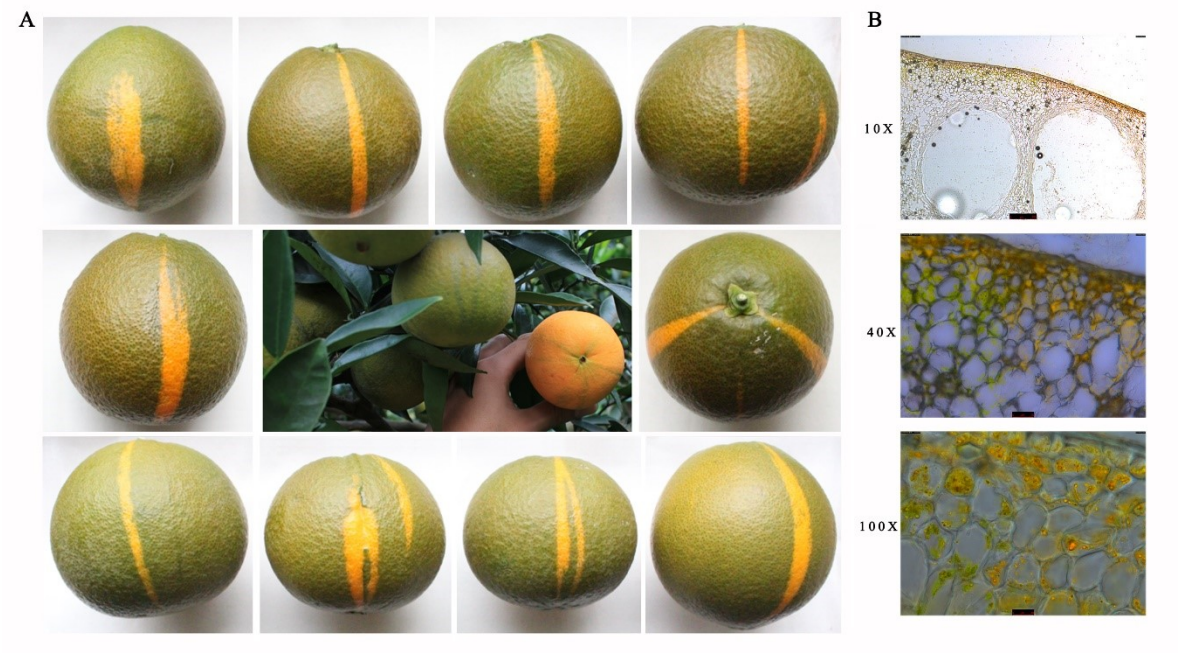
Supplemental Figure S2 Chromatographic profiles of chlorophylls and carotenoids in WT and MT during fruit ripening. (A) Ultra-performance liquid chromatography (UPLC) profiles monitored at 664 nm for chlorophylls extracted from WT (upper) and MT (bottom) flavedos. (B) High-performance liquid chromatography (HPLC) profiles monitored at 450 nm for carotenoids extracted from WT (upper) and MT (bottom) flavedos. The peaks are numbered according to the elution sequence as marked in the upper right.

Supplemental Figure S3



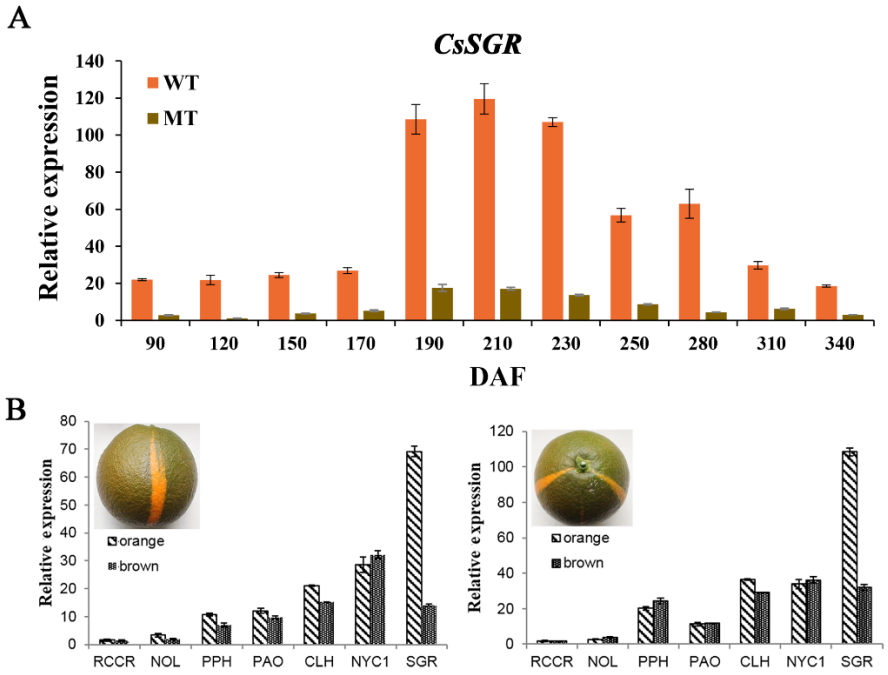
Supplemental Figure S3 Transcriptome comparison of developing flavedos from MT and WT fruits. (A) Venn diagram representing the number of differentially expressed genes (DEGs) between WT and MT at 190, 210, and 230 DAF. (B) Left: Hierarchical cluster and heat map analysis illustrating the expression profiles of the 209 genes that are differentially expressed at all stages as shown in (A); right: Fragments per kilobase million (FPKM) of the candidate gene *CsSGR*. Results are means ± SD from three biological replicates. The color bar indicates the expression levels (represented as FPKM means); red indicates high expression level and blue indicates low expression level. (C) Venn diagram representing shared DEGs among Gene set 1B, Gene set 2 and Gene set 3.

Supplemental Figure S4



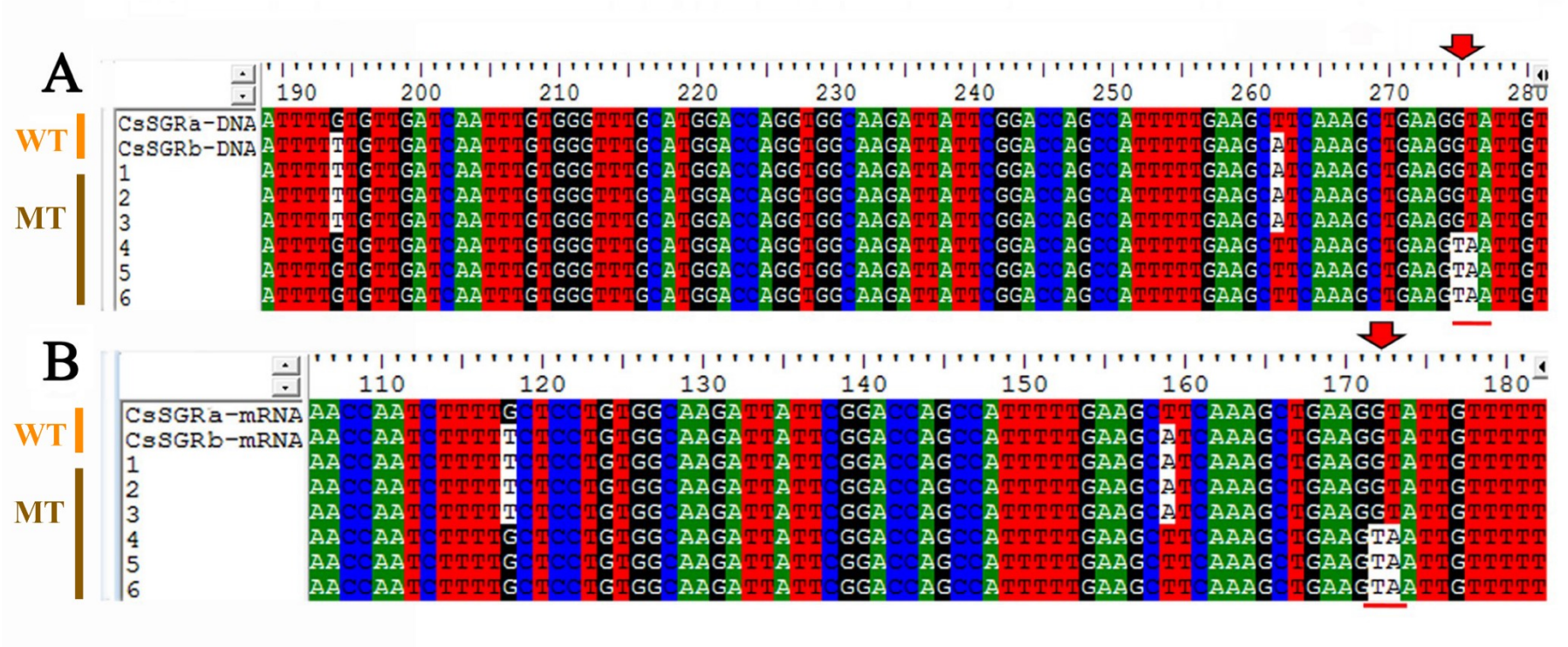
Supplemental Figure S4 Chimeras on MT plants. (A) Phenotypes of various chimeras. (B) Light microscopy of the flavedos of chimeras at various magnifications (10, 40, and 100 times). Top, bar = 250 μm; middle, bar = 25 μm; bottom, bar = 10 μm;

Supplemental Figure S5



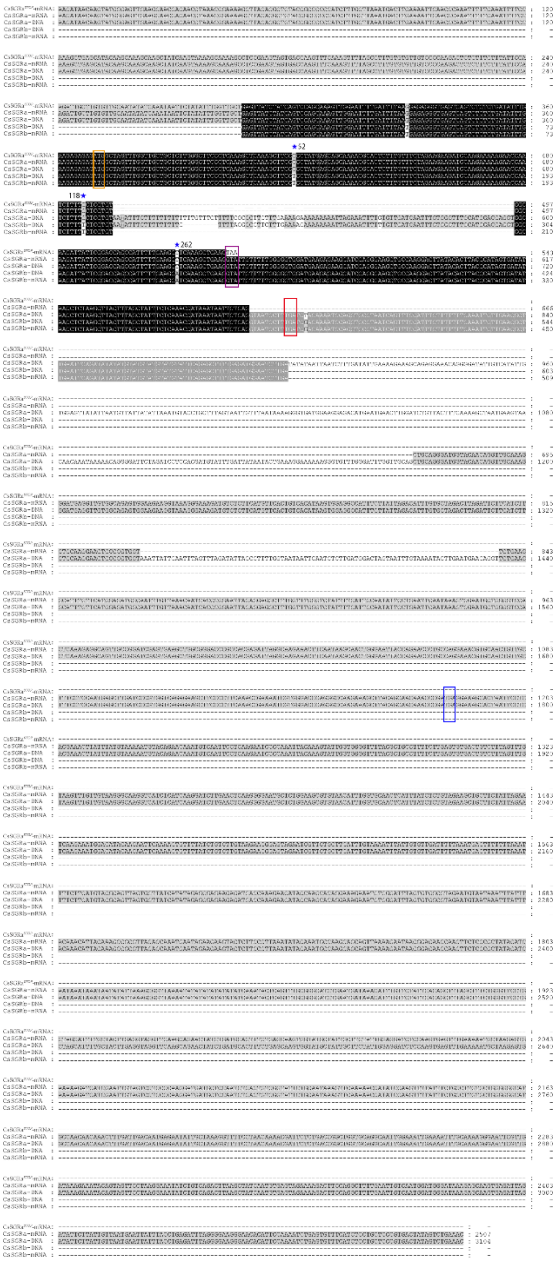
Supplemental Figure S5 Expression profiles of *CsSGR*. (A) *CsSGR* transcript levels measured by RT-qPCR were compared between WT and MT flavedos at various days after flowering (DAF). (B) Differences in transcript levels between *CsSGR* with genes encoding enzymes of the chlorophyll degradation pathway as measured in flavedo sectors (orange or brown) of two chimeric fruit. Abbreviations are as follows: RCCR, red chlorophyll catabolite reductase; NOL, NYC1-like; PPH, pheophytinase; PAO, pheophorbide a oxygenase; CLH, chlorophyllase; NYC1, non-yellow coloring1; SGR, STAY-GREEN. *CsActin* was used for normalization in all RT-qPCR measurements. See Table S14 for PCR primers.

Supplemental Figure S6



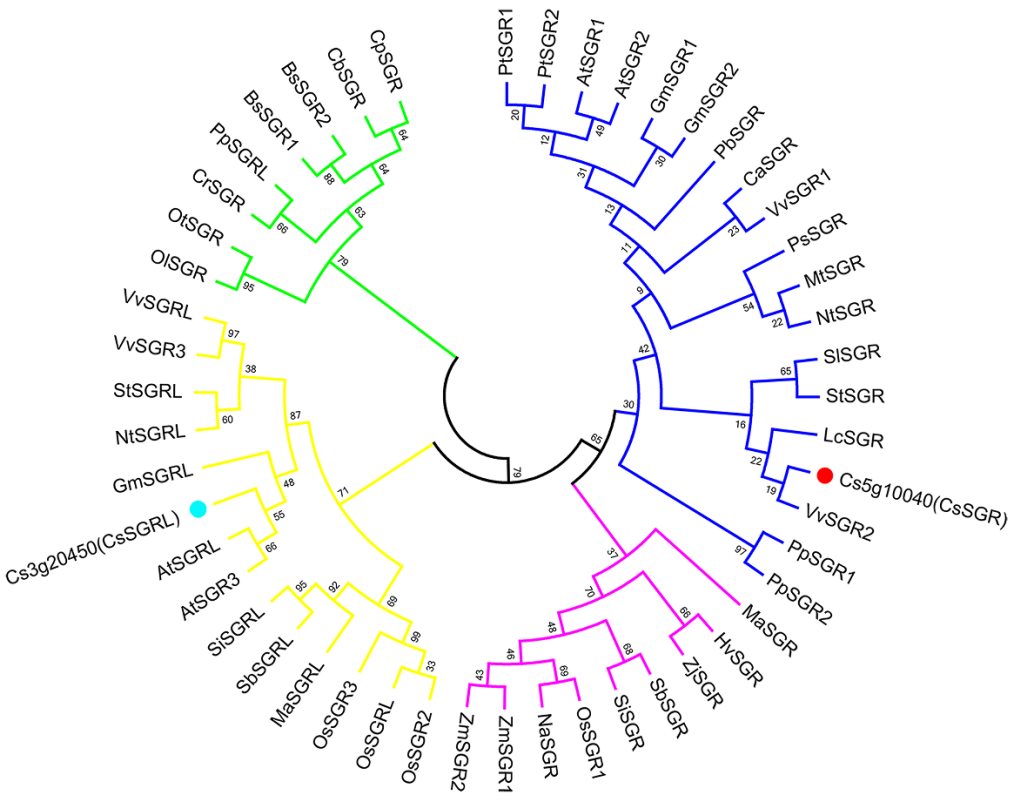
Supplemental Figure S6 PCR-based sequence analysis of MT *CsSGR* alleles compared to WT. (A) PCR-based DNA sequence alignment of *CsSGR* alleles from MT and WT. (B) PCR-based cDNA sequence alignment of MT *CsSGR* alleles compared with WT. Sequences numbered 1, 2, 3 correspond to *CsSGRb*, and 4, 5, 6 correspond to *CsSGRa*. Mutations are marked by red arrows.

Supplemental Figure S7



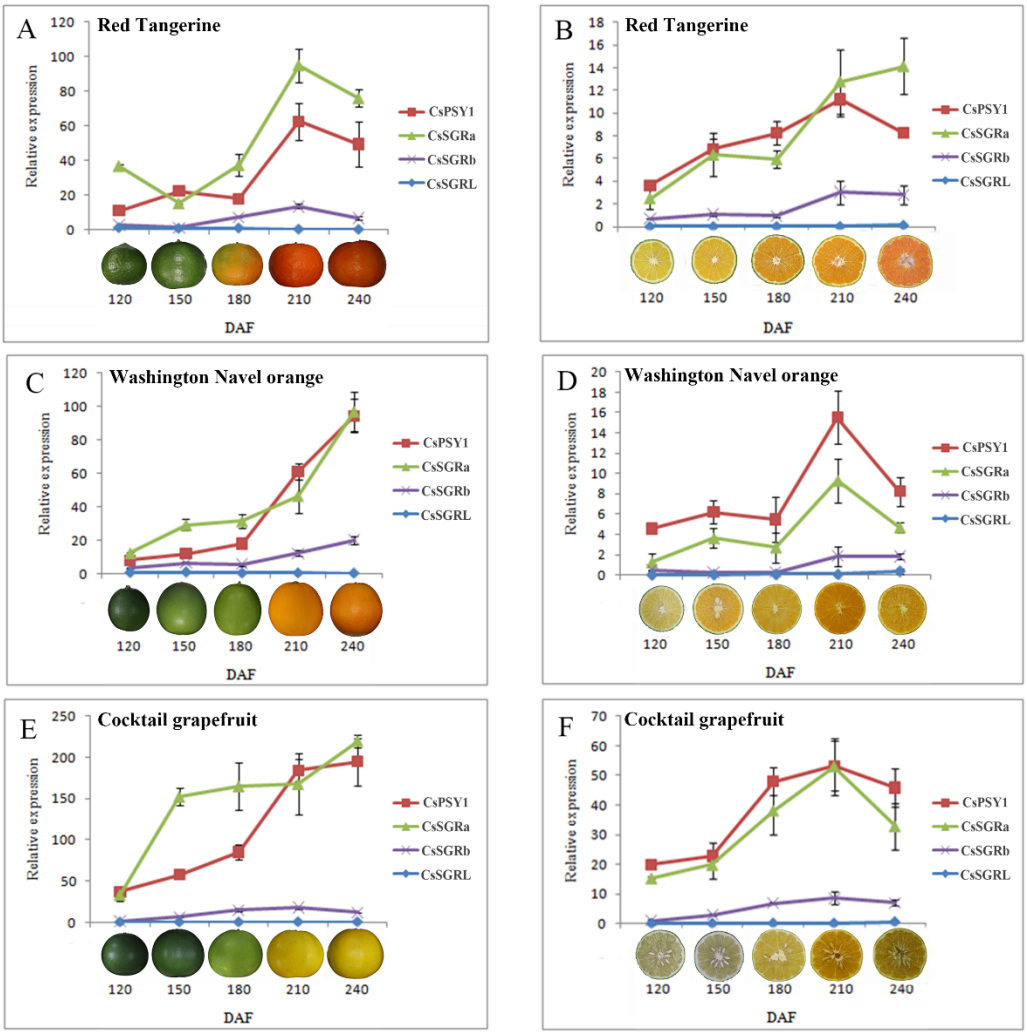
Supplemental Figure S7 Nucleic acid sequence alignment of *CsSGR* alleles. The DNA and mRNA sequence of *CsSGRa*, *CsSGRb* and *CsSGRa*^{STOP} alignments were performed using ClustalW and displayed using the GenDoc program. Positions of the variant nucleotides in *CsSGR* alleles are indicated by blue stars and numbers. The start codons of *CsSGRa*, *CsSGRb* and *CsSGRa*^{STOP} are marked by an orange box. The stop codon of *CsSGRa* is marked by a blue box. The mutation in *CsSGRa*^{STOP} causes a premature stop codon which is marked by a purple box. The mis-spliced region in the *CsSGRb* mRNA results in a premature stop codon as marked by a red box.

Supplemental Figure S8



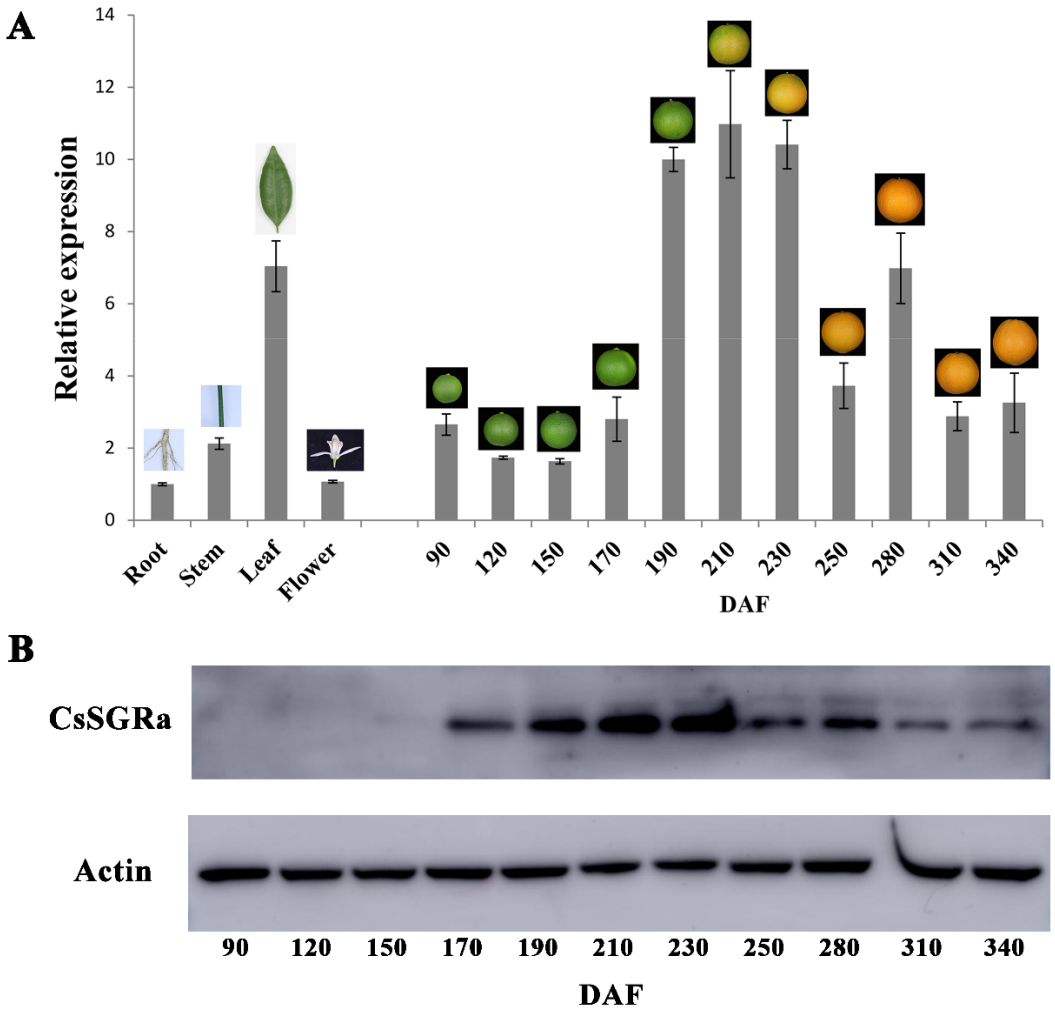
Supplemental Figure S8 Phylogenetic analysis of the SGR and SGRL amino acid sequences. Amino acid sequence alignments were performed using ClustalX. The phylogenic tree was constructed using the Neighbor-joining method and 1,000 bootstraps with the SGR amino acid sequences using MEGA 5.2. Red and azure circles indicate *Citrus sinensis* CsSGR (Cs5g10040) and CsSGRL (Cs3g20450), respectively. The yellow lines indicate the SGRL family. The green lines indicate SGR family members from inferior species. The blue line indicates dicots (except for *Physcomitrella patens* PpSGR1, PpSGR2). The pink line indicates monocots. Abbreviations and protein accession numbers are listed in Supplementary Table S13.

Supplemental Figure S9



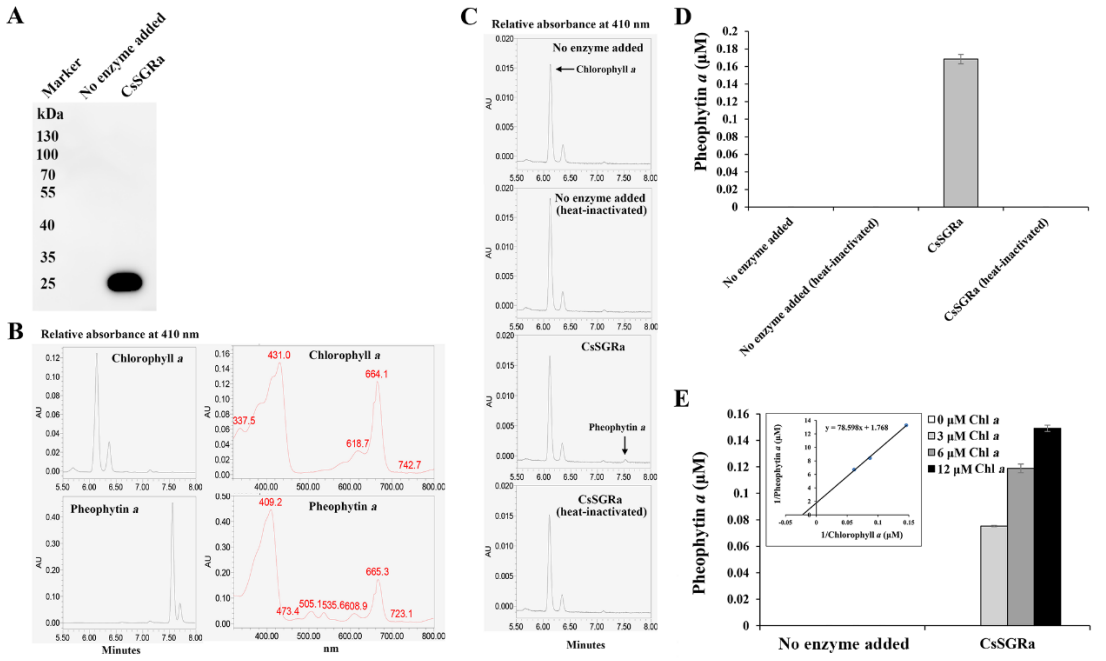
Supplemental Figure S9 Expression profiles of *CsSGRL*, *CsSGRa*, *CsSGRb*, *CsPSY1* in different citrus fruit tissues, developmental stages and species. The expression of these genes was examined by RT-qPCR during fruit development (120, 150, 180, 210, and 240 days after flowering [DAF]) in the flavedo of Red Tangerine (*C. reticulata*) (A), pulp of Red Tangerine (*C. reticulata*) (B), flavedo of Washington Navel orange (*C. sinensis*) (C), pulp of Washington Navel orange (*C. sinensis*) (D), flavedo of Cocktail grapefruit (*C. paradisi*) (E), and pulp of Cocktail grapefruit (*C. paradisi*) (F). All expression values were normalized to the expression values of the *CsActin* gene. Mean and SD values were obtained from three biological replicates. These experiments were repeated at least twice with similar results. The related primers and detailed informations are listed in Supplementary Table S14.

Supplemental Figure S10



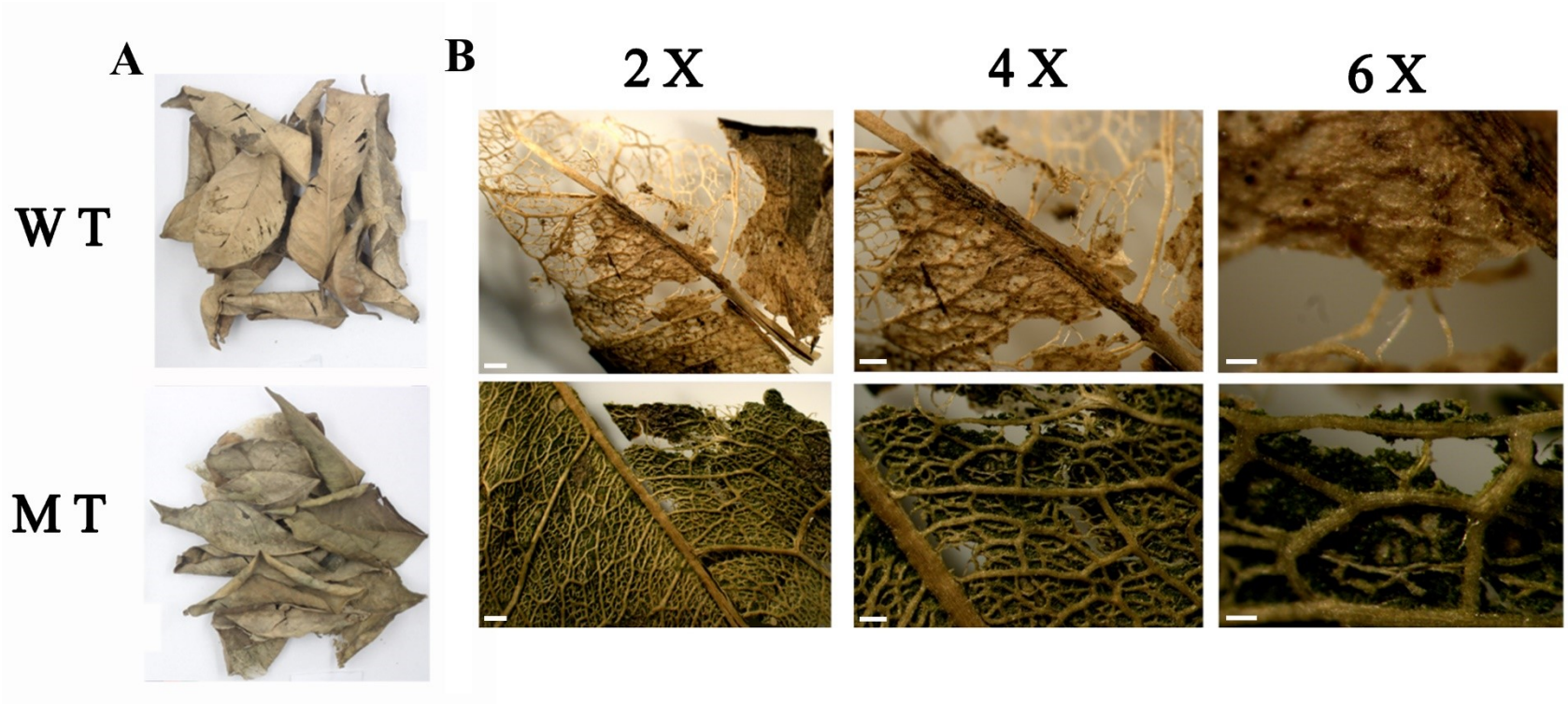
Supplemental Figure S10 Citrus *CsSGRa* transcript and protein levels. (A) Relative levels of *CsSGRa* transcript in various tissues and stages of developing fruit. Mean and SD values were obtained from three biological replicates. (B) Protein levels of CsSGRa at various stages of fruit development. Gene expression values were normalized to the expression values of the actin gene. For immunoblotting, actin served as a loading control and was analyzed from a duplicate gel.

Supplemental Figure S11



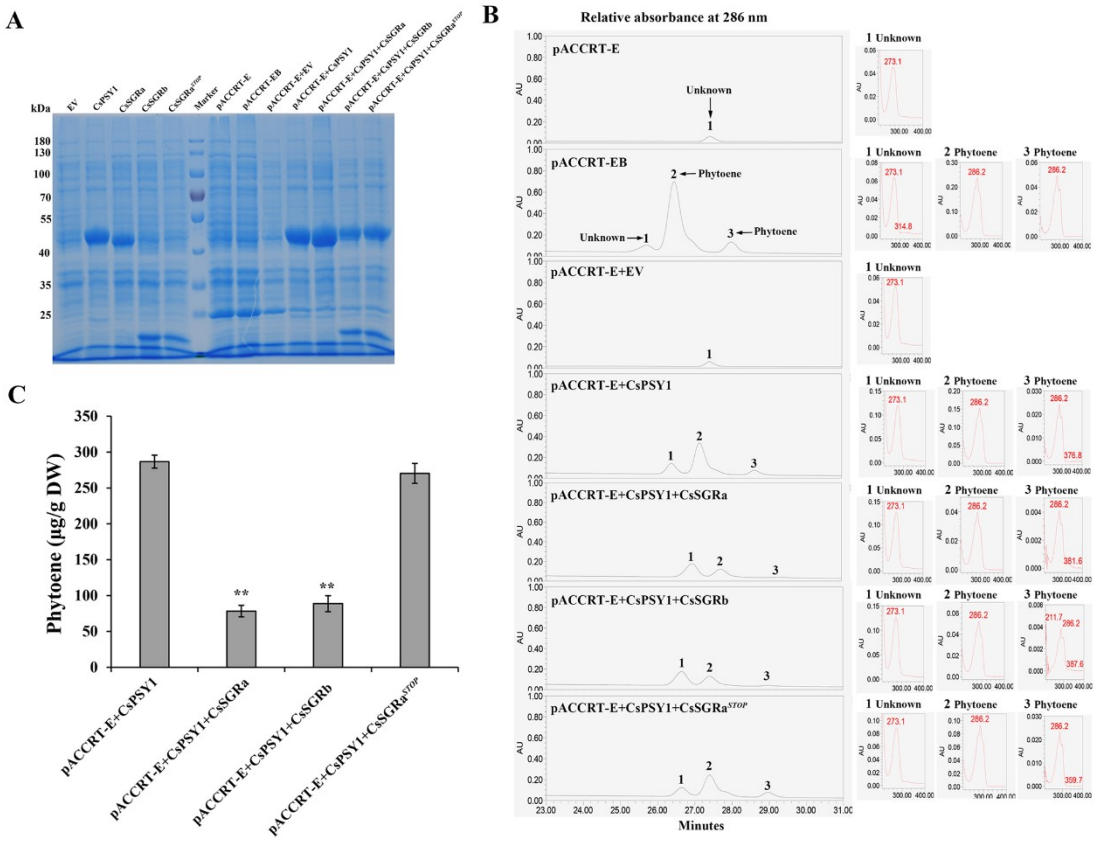
Supplemental Figure S11 Mg-dechelating activity of CsSGRa. Recombinant CsSGRa protein produced in a wheat germ protein expression system, and without further purification, was diluted 4-fold into the Mg-dechelatase reaction buffer including 6 μM final concentration of chlorophyll *a* and incubated for 60 minutes. After incubation, pigments were analyzed using Ultra-performance liquid chromatography (UPLC). Pigments were detected at 410 nm. (A) Immunoblot analysis of CsSGRa protein produced in the wheat germ protein expression system. (B) UPLC chromatograms (left) and spectra (right) of chlorophyll *a* and pheophytin *a* standards. (C) UPLC chromatograms after incubation of chlorophyll *a* with recombinant CsSGRa, no added protein (No enzyme added), or heat-inactivated CsSGRa (CsSGRa (heat-inactivated)). (D) Pheophytin *a* content of samples as shown in the (C). (E) Enzyme kinetic analysis of Mg-dechelating activity of CsSGRa. Varying amounts of chlorophyll *a* were incubated with CsSGRa protein and controls, respectively. The inset shows the Lineweaver-Burk plot of Mg-dechelating activity of CsSGRa. All the above results show means ± SD from at least three biological replicates.

Supplemental Figure S12



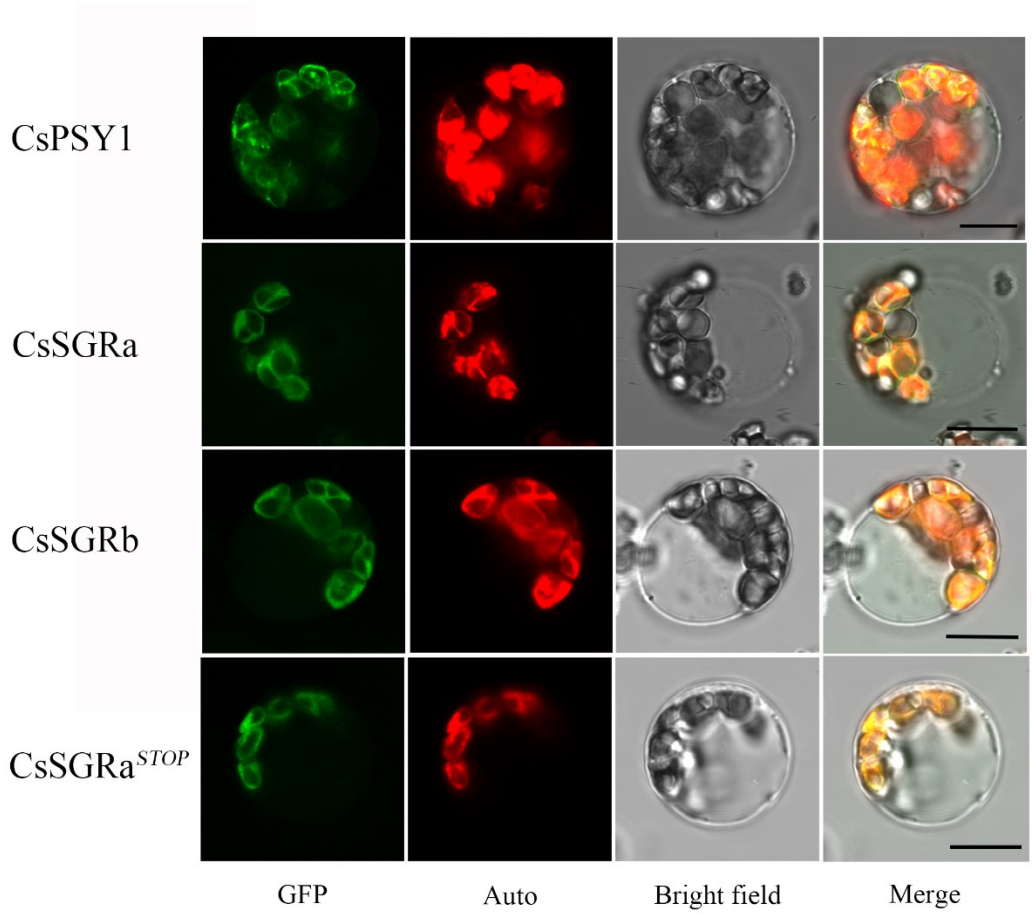
Supplemental Figure S12 Phenotype of withered leaves in WT and MT. Leaves were photographed with a digital SLR camera (A) and observed using a stereomicroscope at the magnifications indicated (B). 2X, bar = 1000 μm; 4X, bar = 500 μm; 6X, bar = 200 μm.

Supplemental Figure S13



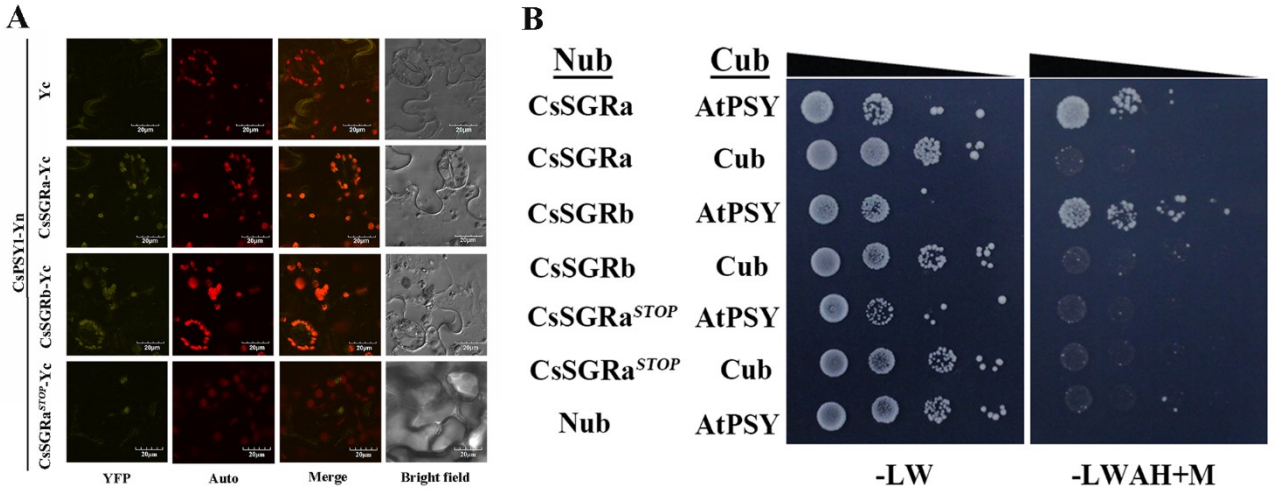
Supplemental Figure S13 Functional analysis of plant CsPSY1 and CsSGR alleles in *E. coli*. *E. coli* transformants harboring pACCRT-E produced geranylgeranyl pyrophosphate (GGPP), and *E. coli* transformants harboring pACCRT-EB produced phytoene. (A) SDS-PAGE gel stained with Coomassie brilliant blue demonstrating the expressed proteins of induced *E. coli* transformants. See the detailed information on the plasmid combinations in Supplementary Table S16. (B) High-performance liquid chromatography (HPLC) carotenoid profiles of *E. coli* transformants. Pigments were detected at 286 nm. (C) Phytoene content of *E. coli* transformants. Results are means ± SD from at least three biological replicates. Asterisks indicate statistically significant differences compared to pACCRT-E+CsPSY1 (Student's *t* test P value, **P<0.01).

Supplemental Figure S14



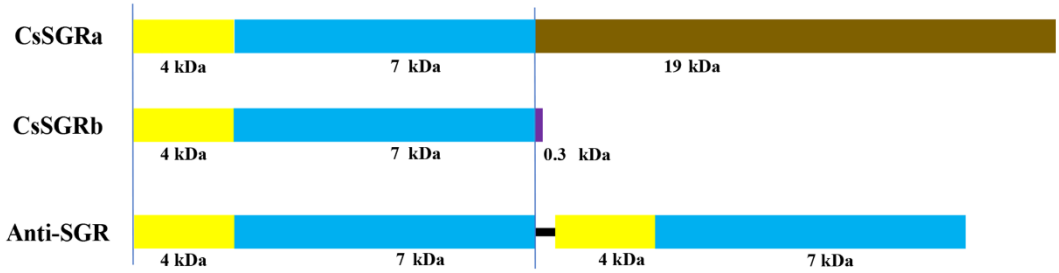
Supplemental Figure S14 Localization of CsSGR-GFP proteins and CsPSY1-GFP. CsSGRa-GFP, CsSGRb-GFP, CsSGRa^{STOP}-*GFP* and CsPSY1-GFP were transiently expressed in citrus leaf protoplasts. Auto, Chlorophyll autofluorescence. Merge, indicates the digital addition of the Auto and GFP signals. Bars = 10 μm.

Supplemental Figure S15



Supplemental Figure S15 Testing interaction between CsSGRa, CsSGRb or CsSGRa^{STOP} with PSY from citrus and Arabidopsis. (A) BiFC analysis. Genes encoding CsSGRa (CsSGRa-Yc), CsSGRb (CsSGRb-Yc) or CsSGRa^{STOP} (CsSGRa^{STOP}-Yc) as C-terminal yellow fluorescent protein (YFP) fusions, were co-transformed with CsPSY1 as an N-terminal YFP fusion (CsPSY1-Yn) into *N. benthamiana* leaves. The empty vector Yc (EV) was co-transformed with CsPSY1-Yn as a negative control. YFP indicates fluorescence of YFP; Auto, indicates chlorophyll autofluorescence; Merge, indicates the digital addition of the Auto and YFP signals. Bars = 20 μm. (B) Y2H analysis. CsSGRa, CsSGRb and CsSGRa^{STOP} interactions with Arabidopsis AtPSY were examined by cotransforming yeast with genes encoding pairs of proteins fused to either the N-terminal (Nub) or C-terminal (Cub) ubiquitin moiety and spotting transformants onto either nonselective (−LW) or fully selective medium containing 150 mM Met (−LWAH +M) in a series of 10-fold dilutions. Empty vectors expressing only Nub and only Cub were used as negative controls.

Supplemental Figure S16



>Anti-SGR nucleotide sequence

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AAGAACCAATCTTTTTCTCCTGTGGCAAGATTATTCGGACCAGCCATTTTTGAAGCATC
AAAGCTGAAGGTATTGTTTTTGGGGGTGGATGAAGAGAAGCATCCAGGGAAGCTGCC
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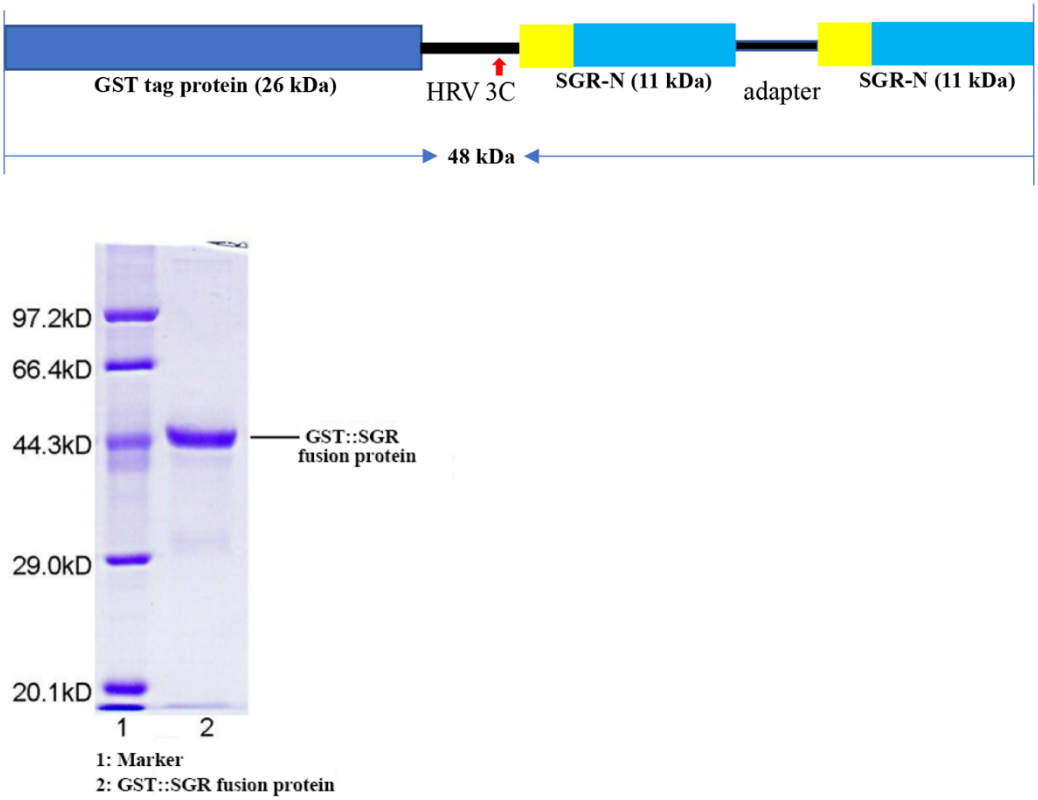
ACCAGCCATTTTTGAAGCATCAAAGCTGAAGGTATTGTTTTTGGGGGTGGATGAAGAG
AAGCATCCAGGGAAGCTGCCAAGGACTTATACACTTACCCATAGTGATATAACCTCTAA
GCTTACTTTAGCTATTTCTCAAACCATAAATAATTCTCAGGTAATCCTTCTCGAGCGGCC
GCATCGTGACTGA

>Anti-SGR protein sequence

GPLGSMASLVAALGLPSKLKASSYEQQNALFVSRRRSKKKNQSFSPVARLFGPAIFEASKL
KVLFLGVDEEKHPGKLPRTYTLTHSDITSKLTLAISQTINNSQVILSSGMASLVAALGLPSKL
KASSYEQQNALFVSRRRSKKKNQSFSPVARLFGPAIFEASKLKVLFLGVDEEKHPGKLPRT
YTLTHSDITSKLTLAISQTINNSQVILLERPHRD-

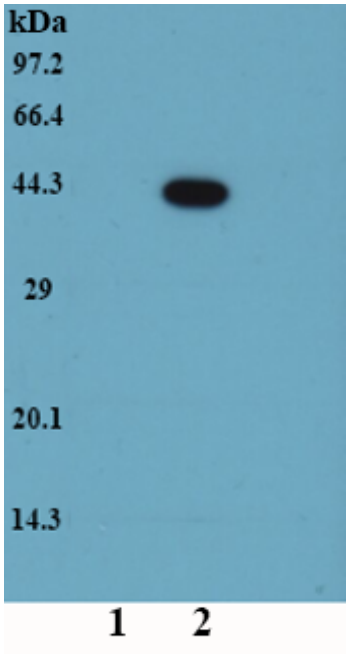
Supplemental Figure S16 The construction of anti-SGR, the peptide used for raising polyclonal antibodies against SGR. The construction of CsSGRa, CsSGRb, and anti-SGR was shown. The sequence shared by CsSGRa and CsSGRb was used to generate an antibody that can detect both CsSGRa and CsSGRb proteins. The colors represent: yellow, the chloroplast transit peptide; blue, the region shared by CsSGRa and CsSGRb; brown, the remaining portion of CsSGRa; purple, the remaining portion of CsSGRb; black, the adapter sequence. The noncolored sequences correspond to the residues remaining after GST cleavage.

Supplemental Figure S17



Supplemental Figure S17 Overexpression of anti-SGR. Top, a cartoon of the construct used to generate the anti-SGR antigen. A conserved sequence shared between SGRa and SGRb, separated by an adapter sequence, was cloned downstream of a GST sequence followed by an HRV 3C cleavage site (the red arrow). Bottom, the anti-SGR fusion protein showed a band similar to the predicted size of 48 kDa.

Supplemental Figure S18



Supplemental Figure S18 The antibody specificity of anti-SGR. Each lane contained 30 μg protein extract in buffer (50 mm Tris-HCl, pH 6.8, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 2% (w/v) SDS, 1% (v/v) β-mercaptoethanol), which was heat-denatured prior to separation on 15% SDS-PAGE and blotting onto PVDF membranes (Millipore NO: ISEQ00010). Membranes were blocked overnight at 4 °C with a TBST solution containing 5% (w/v) not-fat dry milk powder and then incubated with anti-SGR antibodies (1:500 dilution) in the same solution for 2 h at room temperature. After rinsing five times for 10 min each with TBST solution, the membranes were incubated with the secondary antibodies, HRP-labeled goat anti-rabbit IgGs (Frdbio: SAB90000H) at 1:5000 in TBST solution, containing 5% (w/v) not-fat dry milk powder and rinsed six times for 10 min each with TBST solution. The signal was detected using the Enhanced Chemiluminescence (ECL) substrate (Frdbio: WBR0701) and visualized with films by using photographic developer and fixer (Frdbio: WBR0801). Western blotting was performed by the Frdbio company (Frdbio, Wuhan, China). Protein extracts were obtained from: lane 1, control cells carrying the empty vector (pGEX-6p-1); lane 2, cells expressing the anti-SGR fusion protein (pGEX-6p-SGR).