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## Title

Activity examination of plant Mg-dechelataase and its bacterial homolog in plants and *in vitro*

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## Abbreviations

AbSGR-h, *Anaerolineae* SGR homolog; DEX, dexamethasone; HCAR, 7-hydroxymethyl chlorophyll a reductase; LHCII, light-harvesting complex of photosystem II; NYC1, Non Yellow Coloring 1; PPH, pheophytinase; SGR, Stay-Green

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## Abstract

Chlorophyll *a* serves as a photosynthetic pigment in plants. Its degradation is initiated by the extraction of the central Mg by the Mg-dechelataase enzyme, which is encoded by *Stay-Green* (*SGR*). Plant *SGR* is believed to be derived from bacterial *SGR* homolog obtained through horizontal gene transfer into photosynthetic eukaryotes. However, it is not known how the bacterial *SGR* homolog was modified to function in plants. To assess its adaptation mechanism in plants, a bacterial *SGR* homolog derived from the *Anaerolineae* bacterium SM23\_63 was introduced into plants. It was found that the bacterial *SGR* homolog metabolized chlorophyll in plants. However, its chlorophyll catabolic activity was lower than that of plant *SGR*. Recombinant proteins of the bacterial *SGR* homolog exhibited higher activity than those of the plant *SGR*. The reduced chlorophyll catabolic activity of bacterial *SGR* homologs in plants may be associated with low hydrophobicity of the entrance to the catalytic site compared to that of plant *SGR*. This hinders the enzyme access to chlorophyll, which is localized in hydrophobic environments. This study offers insights into the molecular changes underlying the optimization of enzyme function.

## 1. Introduction

Plants degrade chlorophyll during senescence. Proteins of chlorophyll-protein complexes serve as nitrogen sources that should be recovered during senescence (Hörtensteiner and Feller, 2002). Because the chlorophyll-protein complex is resistant to protease activity, chlorophyll should be eliminated before proteolysis. Free chlorophyll produces reactive oxygen species that can damage cells. Active cell participation is crucial for recovering nutrients from the leaves. Therefore, chlorophyll is degraded during senescence (Hörtensteiner, 2004). Chlorophyll degradation is initiated by the extraction of the central Mg (Fig. 1), catalyzed by the Mg-dechelataase enzyme encoded by *Stay-Green* (*SGR* also known as *NYE1*) (Shimoda et al., 2016). This reaction is a crucial step in the chlorophyll-degradation pathway. The crystal structure of bacterial *SGR* homolog has been previously determined (Dey et al., 2022). The catalytic site of this protein was predicted through amino acid substitutions and docking simulations (Dey et al., 2021). The active sites comprised two conserved aspartate residues and one histidine residue. The anticipated catalytic mechanism involves either the coordination of aspartate with Mg and extraction by weakening the coordination between Mg and the nitrogen of the chlorine ring or the coordination of histidine with Mg and extraction using aspartate as a proton donor.

During evolution, organisms have developed novel metabolic pathways. For this, either novel enzymes should be prepared or the pre-existing enzymes should accept novel substrates. Horizontal gene transfer is a common method for obtaining novel enzymes. Enzymes catalyze chemical reactions on specific substrates. However, a single enzyme

can exhibit various catalytic activities called promiscuous activities (Peracchi, 2018). Cellular enzymes do not exhibit promiscuous activity because the substrate for the activity is absent. Additionally, enzymes with low promiscuous activity may not significantly disrupt metabolic systems (Copley et al., 2023). If such enzymatic activity is favorable to other organisms post-transfer, a novel metabolic pathway can be constructed. Based on a phylogenetic tree of amino acid sequences, plant *SGR* are believed to be horizontally transferred from bacteria (Obata et al., 2019). Bacterial *SGR* homolog exhibited Mg-dechelataase activity. However, they are unlikely to be involved in chlorophyll degradation in bacterial cells. The reasons are as follows: (1) The *SGR* homolog has not been found in photosynthetic bacteria. (2) Heterotrophic bacteria having *SGR* homolog could feed on the chlorophyll-protein complex of photosynthetic organisms. However, the bacteria may not have evolved the *SGR* homolog to digest and nourish chlorophyll because bacteria with *SGR* homologs do not have the genes for the chlorophyll-degrading system after Mg is removed. (3) Some *SGR* homologs do not have the amino acids essential for Mg dechelation. These proteins are inactive for Mg dechelation (Obata et al., 2019). (4) Plant *SGRs* have been shown to bind to a variety of proteins. It is possible that bacterial *SGR* homologs also regulate the function of their proteins by binding to other proteins. The possibility that bacteria feed on chlorophyll and use *SGR* homologs to digest it cannot be completely excluded. However, for the above reasons, *SGR* homologs may not catabolize chlorophyll in bacteria cells. Though the primary function of bacterial genes should not be Mg-dechelation, the genes of plants and bacteria have high sequence homology and form a clade in phylogenetic analysis (Obata et al., 2019). Therefore, we call the bacterial gene an *SGR* homolog in this study.

Although it is predicted that plants obtain the *SGR* gene from bacteria for chlorophyll degradation, the modification of bacterial proteins to function efficiently within chloroplasts remains unclear. In this study, we examined plant transformants and recombinant proteins prepared using plant *SGR* and bacterial *SGR* homologs to understand the adaptation process of horizontally transferred genes. This study suggests that plant *SGRs* were more active than bacterial *SGR* homologs in degrading chlorophyll within chloroplasts because plant proteins have a more hydrophobic surface than bacterial proteins and are more accessible to substrates in a hydrophobic environment. This result suggests an adaptive process of genes obtained by horizontal transfer.

## 2. Materials and methods

### 2.1. Cloning of *Arabidopsis SGR1* (*AtSGR1*) and *Anaerolineae SGR* homolog (*AbSGR-h*)

*AtSGR1* (AT4G22920) and *AbSGR-h* (KPK94580), which were optimized for *Escherichia coli* codon usage, were amplified from artificially synthesized DNA (Fig. S1) using primer sets (Table S1) (Obata et al., 2019). The amplified DNA fragments were cloned into the FATT-tag vector through the *NcoI* and *BamHI* sites using the In-Fusion cloning system (Takara Bio) for enzymatic assay (Chen et al., 2017; Obata et al., 2019; Sangawa et al.,

2013). *AtSGR1* transit peptide and *AbSGR-h* were amplified through PCR using specific primer sets (Table S1). The amplified fragments were mixed and further amplified using the *Arabidopsis* transit forward and *AbSGR-h* reverse primer sets. Amplified *AbSGR-h* fragments comprising the *AtSGR1* transit peptide were subsequently cloned into pET30a (+) using the *NdeI* and *XhoI* sites for protein size determination.

## 2.2. *AbSGR-h* expression in *Arabidopsis*

*AbSGR-h* with *AtSGR1* transit peptide were prepared using specific primer sets (Table S1) and inserted into the Gateway entry vector pENTR4 Dual (Invitrogen) between the *NcoI* and *EcoRV* sites. Subsequently, the construct was transferred into the Gateway-compatible inducible vector pOpOn, which was constructed from pOpOff2 through antisense fragment elimination (Craft et al., 2005; Shimoda et al., 2016; Wielopolska et al., 2005). Transgene expression is regulated by the pOp6 promoter. This construct was introduced into *Agrobacterium tumefaciens* (strain GV3101) and transformed into *Arabidopsis thaliana* (Columbia ecotype). Transgenic plants were cultivated for 4 weeks at 24°C under continuous light conditions (100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). To induce transgene expression, the plants were sprayed with 10  $\mu\text{M}$  dexamethasone (DEX) supplemented with 0.05% (v/v) Silwet L-77. Control plants were sprayed with water containing 0.05% Silwet L-77 without DEX.

## 2.3. *AbSGR-h* expression in *Nicotiana benthamiana* (*N. benthamiana*)

SGRs were transiently overexpressed in *N. benthamiana* leaves through *Agrobacterium* infiltration (Park et al., 2007). The constructs used for *Arabidopsis* transformation were used for transient overexpression. DEX was administered during *Agrobacterium* infiltration. Three days post-infiltration, pigments and proteins were examined. The green fluorescent protein (GFP)-fusion *AbSGR-h* was generated from PCR-amplified *AbSGR-h* with *AtSGR1* transit peptide and GFP fragments using specific primer sets (Table S1). It was subsequently inserted into the pGreenII vector (Hellens et al., 2000) at the *NcoI* site under the control of the 35S promoter. Four days post-infiltration, protoplasts were obtained by incubating the cut leaves with enzyme solution (0.6 M mannitol, 1% [w/v] cellulase, 0.2% [w/v] macerozyme, 470 mM KCl, 50 mM  $\text{CaCl}_2$ , and 10 mM MES-KOH; pH 5.6) for 5 h. The imaging data was obtained using a confocal laser scanning microscope (LSM980, Zeiss) at an excitation and emission wavelength of 488/490-579 nm for GFP and 655/641-694 nm for chlorophyll, respectively.

## 2.4. Pigment analysis

Pigments were extracted from leaf discs (0.25  $\text{cm}^2$ ) using 250  $\mu\text{L}$  acetone. Subsequently, they were separated and quantified through high-performance liquid chromatography (HPLC) using a C18 column (4.6 $\times$ 100 mm; Poroshell 120 EC-C18, Agilent) with an eluent (methanol:acetonitrile:acetone [1:2:1 v:v:v]) at a flow rate of 1  $\text{mL min}^{-1}$  at 40°C. Pigments

were monitored using a diode array detector at 663, 648, and 664 nm absorbance for chlorophyll *a*, *b*, and pheophytin *a*, respectively.

## 2.5. Immunoblotting analysis

Each leaf disc (0.25 cm<sup>2</sup>) was frozen in liquid nitrogen and disrupted by shaking with zirconia beads. Proteins were extracted using 250 µL protein extraction buffer (125 mM Tris-HCl, 2% [w/v] sodium dodecyl sulfate [SDS], 5% [w/v] sucrose, and 5 % [v/v] 2-mercaptoethanol; pH 6.8). Following centrifugation (22,500 *g*, 5 min), the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane for immunoblotting (Shimoda et al., 2016). FLAG-tagged proteins were detected using an antibody against FLAG-tag (Sigma-Aldrich). The primary antibodies were diluted with an immunoreaction enhancer solution (Can Get Signal, Toyobo). Anti-mouse IgG conjugated to horseradish peroxidase (HRP) was used as the secondary antibody. Histidine-tagged proteins were detected using an antibody against histidine-tag (Anti-His-tag mAb-HRP-Direct T; MBL). The HRP activity was visualized using Agrisera ECL Bright (Agrisera).

## 2.6. Enzyme assay

Protein expression plasmids were introduced into *E. coli* BL21 (DE3) cells and cultured. The recombinant protein was expressed in an auto-induction medium at 37°C for 16 h with shaking at 120 rpm (Studier, 2005). The culture (1 mL) was centrifuged and the cell pellet was resuspended in 50 µL of BugBuster Protein Extraction Reagent containing 0.1 µL benzonase (Sigma-Aldrich). The lysate was subsequently centrifuged at 22,500 *g* for 5 min. The resulting supernatant was used as an enzyme solution. The light-harvesting complex of photosystem II (LHCII) was prepared as previously described (Shimoda et al., 2012). *E. coli* lysate (20 µL) was incubated with chlorophyll *a* (840 pmol) or LHCII (810 and 780 pmol of chlorophyll *a* and *b*, respectively) at 25°C for 1 h, and pigments were analyzed through HPLC.

## 2.7. Substrate docking analysis

X-ray crystal structures of AbSGR-h (PDB:7Y5Y) (Dey et al., 2022) and 7-hydroxymethyl chlorophyll *a* reductase (HCAR) (PDB:SDQR) (Wang and Liu, 2016) were used for docking simulations. The AtSGR1 protein structure was predicted using the Swiss-Model (Waterhouse et al., 2018) with AbSGR-h as the template. Chlorophyll *b* reductase encoded by *Non Yellow Coloring 1* (NYC1) and pheophytinase (PPH) structures were obtained from the AlphaFold Protein Structure Database (Varadi et al., 2022). Predicted transit peptides were eliminated. The chlorophyll derivative structures were prepared using Chem3D (Hulink). A protein-ligand docking study was performed using AutoDock Vina (Trott and Olson, 2010). Interactions in the docked conformations were visualized using the PyMOL software (Delano W, 2002).

### 3. Results

#### 3.1. Bacterial SGR homolog expression in plants

Plant SGR genes were believed to be horizontally transferred from bacteria. Recombinant bacterial SGR homologs exhibit Mg-dechelataase activity (Dey et al., 2021; Dey et al., 2022; Obata et al., 2019). However, their activities in plants remain unclear. To assess their activity in plants, *Anaerolineae* bacterium SM23\_63 SGR homolog (*AbSGR-h*) was introduced into *Arabidopsis thaliana*. A chloroplast transit peptide from *Arabidopsis* SGR1 (*AtSGR1*) was attached to the N-terminus of *AbSGR-h*. The constitutive overexpression of SGR degrades chlorophyll and inhibits plant growth (Park et al., 2007). Therefore, the DEX induction system was used to analyze *AbSGR-h* activity in plants. Two independent *AbSGR-h* transformants (*AbSGR-h*\_#5 and #6) were generated and compared to the *AtSGR1* transformant used in a previous study (Shimoda et al., 2016). Following DEX treatment, *AbSGR-h*-transfected plant leaves turned yellow (Fig. 2A). To assess their levels, the proteins were subjected to immunoblotting (Fig. 2B). Both *AtSGR1* and *AbSGR-h* have FLAG tags at their C-termini, which were used to assess the protein levels. The calculated molecular sizes of *AtSGR1* and *AbSGR-h* containing FLAG tag were 25.7 kDa and 19.0 kDa, respectively. The corresponding size bands of *AtSGR1* were observed as indicated by red arrowheads (Fig. 2B), indicating *AtSGR1* accumulation following DEX treatment. In *AtSGR1*-expressing plants, strong bands were observed at approximately 40 kDa as indicated by cyan arrowheads. It is unlikely that these bands represent *AtSGR1* with a transit peptide because the calculated molecular size of the corresponding band is 31.0 kDa. *AtSGR1* recombinant proteins are reported to create the dimeric complex by forming a disulfide bond (Xie et al., 2019), which may correspond to the large molecular weight band observed after *AtSGR1* induction. However, this band remained after treatment with reducing agents, which differs from previous studies. The nature of these bands remains unclear. We cannot exclude the possibility that these large proteins have enzymatic activities. The expressed *AbSGR-h* bands were approximately 16 kDa, which was smaller than the calculated protein size (19.0 kDa). Larger bands (25 kDa) were detected, possibly *AbSGR-h* with transit peptides. The calculated molecular size of *AbSGR-h* containing the transit peptide is 24.4 kDa. To examine whether these two bands represented *AbSGR-h* with or without a transit peptide, recombinant *AbSGR-h* proteins were prepared and subjected to immunoblotting. Both recombinant proteins had FLAG tags and the same amino acid sequences expressed in transformed plants. Immunoblotting indicated that the band sizes in *AbSGR-h*-overexpressing plants were similar to those in *AbSGR-h* with or without a transit peptide. Therefore, a smaller band of approximately 16 kDa was identified as mature *AbSGR-h*. The presence of *AbSGR-h* without a transit peptide indicates the successful accumulation of mature *AbSGR-h* in chloroplasts, facilitating chlorophyll degradation.

To further determine the localization of *AbSGR-h* within the chloroplast, GFP was fused to

the C-terminus of AbSGR-h and expressed in *N. benthamiana* leaves through *Agrobacterium*-mediated infiltration. Four days post-injection, the protoplasts were isolated and observed under a microscope (Fig. 2C). In the area infiltrated with AbSGR-h-GFP fusion gene, GFP fluorescence co-localized with chlorophyll fluorescence. In contrast, GFP fluorescence was observed outside chloroplasts when GFP was expressed alone. These observations indicate that AbSGR-h accumulates in chloroplasts.

Chlorophyll contents of the transformants were determined (Fig. 2D). AtSGR1-induced plants successively degraded chlorophyll, as reported previously (Shimoda et al., 2016). AbSGR-h-induced transformants also degraded chlorophyll. This indicates that bacterial SGR homologs have catalytic activity in chloroplasts. Chlorophyll *a* and *b* levels were reduced in both AtSGR1- and AbSGR-h-induced transformants. The AbSGR-h transformant exhibited less chlorophyll degradation compared to AtSGR1. Chlorophyll *a* was significantly degraded on the initial day of AtSGR1 induction, resulting in a reduced chlorophyll *a/b* ratio in the transformants. However, following DEX treatment, the AbSGR-h transformants did not exhibit a significant reduction in the chlorophyll *a/b* ratio. Based on our observations, bacterial AbSGR-h possibly degrades chlorophyll in chloroplasts.

### 3.2. Transient expression of SGRs in *N. benthamiana*

In *Arabidopsis* transformants, a high molecular weight band was detected when AtSGR1 was induced. Since it was unclear whether this large protein complex is an active enzyme or not, it was difficult to compare the protein level and chlorophyll-degradation rate within the chloroplast. Next, the transient expression of AtSGR1 and AbSGR-h in *N. benthamiana* leaves was used to compare their activities. *Agrobacterium* with plasmids prepared for *Arabidopsis* transformation were used. DEX was administered upon *Agrobacterium* injection into *N. benthamiana* leaves. Following 3 d of incubation, yellowing occurred in the areas expressing AtSGR1 and AbSGR-h (Fig. 3A). Protein expression was determined using immunoblotting (Fig. 3B). Both AtSGR1 and AbSGR-h were observed. The AbSGR-h band intensity was significantly higher than that of AtSGR1, indicating higher AbSGR-h accumulation in *N. benthamiana* leaves. Additionally, no large-sized signal (Fig. 2B) was observed in AtSGR1 overexpressing area. The chlorophyll content was found to be reduced (Fig. 3C), as observed in the *Arabidopsis* transformants. Chlorophyll *a* levels were lower in AtSGR1 expressing area compared to those in expressing AbSGR-h. Chlorophyll *b* levels were reduced in both areas. Therefore, the AtSGR1 expressing area exhibited a lower chlorophyll *a/b* ratio than that of the AbSGR-h-expressing area. Higher protein levels and lower chlorophyll reduction in the AbSGR-h-expressing area indicated that AbSGR-h catalytic activity is low in the *N. benthamiana* chloroplasts. The high molecular weight band in the *Arabidopsis* transformant made it difficult to compare the amount of protein and chlorophyll-degradation rate. However, as no large bands were observed in *N. benthamiana*, AtSGR1 is suggested to be more active in chlorophyll degradation in chloroplasts compared to AbSGR-h.

### 3.3 Enzymatic activities of the recombinant proteins

When expressed in leaves, AtSGR1 metabolized more chlorophyll than AbSGR-h. To examine their enzymatic activities *in vitro*, the recombinant proteins were prepared. The FATT vector was used to generate recombinant AtSGR1 (Obata et al., 2019). The FATT vector has an acidic, disordered-structure polypeptide attached to the N-terminus to increase the solubility of the expressed protein (Sangawa et al., 2013). To our knowledge, this is the only vector that has successfully expressed the plant SGR protein in *E. coli*. AbSGR-h was also cloned into the FATT vector to assess its activity under identical conditions. Because of low AtSGR1 expression levels and unsuccessful purification of active AtSGR1, *E. coli* lysates were used as enzyme solutions. The FATT vector facilitated histidine tag addition on the expressed protein. In expression systems using *E. coli*, the recombinant proteins produced from bacteria may be much higher than those from eukaryotes. Therefore, protein concentrations were normalized using immunoblotting with a histidine tag (Fig. S2). The AbSGR-h expression levels were significantly higher than those of AtSGR1. To normalize the signal intensities of the histidine tag, the *E. coli* lysate containing AbSGR-h protein was diluted 160-fold with lysis buffer. When chlorophyll *a* was incubated with *E. coli* lysates containing AtSGR1 or AbSGR-h, pheophytin *a* accumulated (Fig. 4). The AbSGR-h-containing lysate accumulated higher levels of pheophytin *a*, indicating a higher Mg-dechelataase activity in AbSGR-h *in vitro*. The native substrates of SGR in chloroplasts are incorporated into the chlorophyll-protein complex. To assess the activity of recombinant proteins to the chlorophyll bound to chlorophyll-protein complex, isolated LHCII was incubated with recombinant protein-containing *E. coli* lysates. Following incubation, pheophytin *a* was observed in both reaction mixtures, with higher levels in the AbSGR-h-containing lysate mixture. Collectively, these observations indicated that the activity of the recombinant AbSGR-h was higher than that of AtSGR1.

### 3.4. Protein structures of AtSGR1 and AbSGR-h

AtSGR1 degraded more chlorophyll within the chloroplasts than AbSGR-h. In contrast, the AbSGR-h recombinant protein exhibited a higher activity than AtSGR1 *in vitro*. These observations indicate AtSGR1 optimization for chlorophyll degradation within chloroplasts. In chloroplasts, chlorophyll is incorporated into the chlorophyll-protein complex, which is embedded in the thylakoid membrane. Therefore, chlorophyll localizes to the hydrophobic environment of chloroplasts. When SGR catabolizes chlorophyll, SGR should enter the hydrophobic environment. This indicates that SGR contains hydrophobic regions on its surface. To assess hydrophobicity, AtSGR1 and AbSGR-h structures were analyzed (Fig. 5). Docked substrates were used to identify the catalytic sites. Hydrophobicity at the entrance of the AtSGR1 catalytic site was higher than that of AbSGR-h. When examining the enzymatic activity of recombinant proteins, we used detergent to dissolve chlorophyll and LHCII. Then, the enzyme would be able to contact and catalyze reactions with these substrates regardless of whether the surface of the enzyme is hydrophilic or hydrophobic.

However, in chloroplasts, the hydrophobic regions surrounding the catalytic site facilitate AtSGR1 access to its substrate more efficiently than AbSGR-h. In summary, plant SGR is supposed to efficiently catabolize the thylakoid membrane-embedded chlorophyll by increasing the hydrophobicity surrounding the catalytic site.

## 4. Discussion

### 4.1. Structure change of bacterial SGR homolog to function in chloroplasts

Certain enzymes exhibit promiscuous activities that facilitate the metabolism of substrates that differ from their native substrates (Khersonsky and Tawfik, 2010). This activity can be used to develop novel metabolic pathways. In the case of chlorophyll metabolism, ferredoxin-dependent 3,8-divinyl chlorophyllide reductase of cyanobacteria involved in chlorophyll biosynthesis demonstrates HCAR activity involved in the chlorophyll *b* degradation process though cyanobacteria do not have chlorophyll *b* (Ito and Tanaka, 2014; Lim et al., 2019). When plants acquired chlorophyll *b* and began degrading it, they used this reductase as HCAR (*Arabidopsis*, AT1G04620) and generated a novel enzyme for chlorophyll biosynthesis, which is NADPH-dependent 3,8-divinyl chlorophyllide reductase (AT5G18660). The bacterial SGR homolog used in this study is derived from a non-chlorophyll-producing bacterium. Although the bacterial SGR homolog function within bacteria remains unclear, it is unlikely to degrade chlorophyll as described in the Introduction. Therefore, the Mg-dechelataase activity of bacterial SGR homolog may be the promiscuous activity. There are hydrophobic amino acid residues located at the entrance of the AbSGR-h catalytic site. This hydrophobicity is higher in AtSGR1. It can be assumed that the bacterial SGR homolog does not metabolize substrates in a highly hydrophobic environment, such as within the plasma membrane in bacteria cells. Inserting this gene into ancestral plant cells through horizontal gene transfer requires a chloroplast transit peptide to facilitate chlorophyll metabolism. However, this is inadequate. Modifying the entrance to the catalytic site to enhance hydrophobicity is necessary to access the substrates staying within the thylakoid membrane. This study indicates that when enzyme properties change during evolution, the catalytic site and its entrance that meets the substrate should be optimized depending on substrate localization. The structures of NYC1, HCAR, and PPH proteins involved in chlorophyll degradation show that the entrance to the catalytic site is hydrophobic for all proteins (Fig. 5). HCAR has a high amino acid sequence and protein structure homology with the F<sub>420</sub>-reducing [NiFe]-hydrogenase complex found in methanogenic archaea, possibly indicating its ancestral form (Wang and Liu, 2016). The catalytic site entrance of hydrogenase is not hydrophobic because its substrate is water soluble (Vitt et al., 2014). When this gene was introduced into ancestral photosynthetic organisms to metabolize hydrophobic chlorophyll, the protein was probably modified to become hydrophobic.

### 4.2. Association of AbSGR-h substrate specificity with a decrease in chlorophyll *b*

AbSGR-h metabolizes both chlorophyll *a* and *b*, whereas AtSGR1 only metabolizes chlorophyll *a* *in vitro* (Obata et al., 2019). When AtSGR1 was expressed in *Arabidopsis* and *N. benthamiana*, both chlorophyll *a* and *b* levels were reduced (Fig. 2D and Fig. 3C). Chlorophyll *b* degradation is not directly catalyzed by AtSGR1. When chlorophyll *a* is degraded through AtSGR1 induction, NYC1 is induced and chlorophyll *b* is degraded (Ono et al., 2019; Sato et al., 2018). Therefore, chlorophyll *b* degradation was slower, and the chlorophyll *a/b* ratio was low when AtSGR1 was induced. We expected that chlorophyll *b* would be significantly degraded when AbSGR-h was expressed in *Arabidopsis* and *N. benthamiana*. However, the chlorophyll *b* degradation was smaller in *Arabidopsis* and comparable in *N. benthamiana* to that observed when AtSGR1 was expressed. Since chlorophyll *a* was degraded less than when AtSGR1 was expressed, it is possible, but not conclusive, that the relative chlorophyll *b* degradation rate was higher in AbSGR-h expressing cells. Mg dechelation of chlorophyll *b* by AbSGR-h in plants remains to be experimentally verified. AbSGR-h-associated chlorophyll *b* degradation has the potential to induce photodamage because of the inadequate degradation of chlorophyll *b* catabolites resembling chlorophyll *b* over-producing transformants (Shimoda et al., 2012). As we cannot determine the chlorophyll *b* catabolite and photodamage, the negative effects of the broad substrate specificity of AbSGR-h in plants remain unclear.

#### 4.3. Active form and correct localization of AbSGR-h

In this study, AtSGR1 and AbSGR-h activities were assessed both *in vivo* and *in vitro*. The bacterial SGR homolog-transformed plants degraded less chlorophyll, despite having a higher amount of protein in *N. benthamiana*. In contrast, when the recombinant protein was produced in *E. coli*, the bacterial SGR homolog exhibited greater activity. These findings indicate that bacterial SGR homologs are less active within chloroplasts and are not adapted to chlorophyll metabolism in the photosystem. Additionally, it was hypothesized that the entrance of the SGR homolog to the catalytic site was not hydrophobic and could not access substrates in a hydrophobic environment. To provide this hypothesis, it was assumed that all the synthesized proteins exhibited an appropriate form and enzymatic activity. However, the possibility of incorrect structures affecting the activity cannot be dismissed. To examine the recombinant protein enzymatic activities, we used the soluble fraction of *E. coli* lysate. This solution does not contain the unfolded-insoluble recombinant protein. The properly folded proteins should be contained in the lysate used for the enzymatic reaction. The expression of the bacterial SGR homolog in *Arabidopsis* revealed a signal at approximately 16 kDa, corresponding to a protein within the chloroplast where the chloroplast transit peptide was eliminated. Additionally, transient expression in *N. benthamiana* confirmed the accumulation of AbSGR-h within chloroplasts. These results exclude the possibility that AbSGR-h cannot enter chloroplasts, resulting in its lower activity in chlorophyll degradation. Plant SGRs and AbSGR-h are synthesized in the cytosol and transferred into chloroplasts. Therefore, both protein structures were constructed within the chloroplasts after passing through the chloroplast envelope. There is no apparent reason to

consider that bacterial SGR homologs are less likely to form an appropriate structure than plant SGRs. Based on these considerations, it is predicted that bacterial SGR homologs are less active in chlorophyll metabolism because they cannot access substrates in the chloroplasts. The *Arabidopsis* SGR1 sequence used in this study yielded low amounts of recombinant protein, despite the sequence being optimized for expression in *E. coli*. Bacterial SGR homologs and *Chlamydomonas* SGR are readily expressed (Dey et al., 2022; Matsuda et al., 2016), and a certain amount of *Arabidopsis* SGR-like protein (AT1G044000) is also expressed in *E. coli* (Obata et al., 2019). It remains unclear why plant SGRs are expressed in lower levels in *E. coli*. For a detailed analysis of plant SGRs, it is necessary to establish a mass expression system in *E. coli*.

## 5. Conclusion

In this study, we examined the activity of plant and bacterial SGR homologs both in plants and *in vitro*. Plant SGR and bacterial SGR homologs exhibited higher activity in plants and *in vitro*, respectively. Structural analysis demonstrated that hydrophobicity at the entrance of the catalytic site was higher in plant SGR. These observations indicated that the bacterial SGR homolog modified the surface hydrophobicity to access the hydrophobic environment-localized substrate to become the plant SGR (Fig. 6). This study offers a hypothesis for the production of novel enzymes by exploiting their versatile activity. It was reported that bacterial SGR homologs metabolize chlorophyll *b* and other chlorophyll derivatives, whereas plant SGR does not (Obata et al., 2019). The effect of the broad substrate specificity of bacterial SGR homologs in plants remains unclear.

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## Figure legends

### Figure 1. Mg extraction by Mg-dechelataase

The central Mg of chlorophyll *a* is extracted by Mg-dechelataase, which is encoded by *Stay-Green* (SGR). Two protons are inserted to form pheophytin *a*.

### Figure 2. Chlorophyll degradation and protein accumulation through DEX treatment

(A) AtSGR1 and two genetically independent AbSGR-h transformants (AbSGR-h\_#5 and #6) are cultured for 4 weeks. Subsequently, they are treated with DEX for 2 d. (B) Proteins are extracted from leaf discs. The results are normalized to the leaf area. FLAG-tagged proteins are observed using an anti-FLAG antibody. Red arrows indicate mature AtSGR1 and AbSGR-h, respectively. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)

large subunit is stained with Coomassie Brilliant Blue (CBB). (C) Green fluorescent protein (GFP)-fused AbSGR-h is transiently expressed in *N. benthamiana* leaves through *Agrobacterium* infiltration. After 4 d, protoplasts are isolated and GFP and chlorophyll fluorescence are observed. Mixed fluorescence images are used as merged images. Bars = 10  $\mu$ m. (D) Chlorophyll content and chlorophyll *a/b* ratio are examined with or without DEX treatment. Chlorophyll is extracted from the leaves at 0, 1, and 2 d following DEX treatment. Chlorophyll content is normalized to the leaf area. Error bars represent the standard deviation (SD) (n = 3). \*  $p < 0.05$  and \*\*  $p < 0.01$  (*t*-test).

Figure 3. Transient overexpression of AtSGR1 and AbSGR-h

(A) *N. benthamiana* is infiltrated through recombinant *Agrobacterium* containing DEX-inducible AtSGR1 and AbSGR-h. *Agrobacterium* without the vectors is used as the empty control. Infiltrated leaves are cultured for 3 d. (B) Proteins are extracted from leaf discs. The results are normalized to the leaf area. FLAG-tagged proteins are observed using an anti-FLAG antibody. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit is stained with Coomassie Brilliant Blue (CBB). (C) Chlorophyll content and chlorophyll *a/b* ratio after 3 d of *Agrobacterium* infiltration are examined. Chlorophyll content is normalized to the leaf area. Different letters indicate significant differences at  $p < 0.01$  through the *t*-test. Error bars represent the standard deviation (SD) (n = 3).

Figure 4. Enzymatic activity of recombinant AtSGR1 and AbSGR-h

*Escherichia coli* lysate expressing AtSGR1 or AbSGR-h is used as the enzyme solution. Recombinant protein concentration is normalized to the immunoreaction signal intensities of the histidine tag. *E. coli* lysate with empty vector (control), AtSGR1 or AbSGR-h expressing vector is incubated with chlorophyll *a* or light-harvesting complex of photosystem II (LHCII).

Figure 5. Structure of chlorophyll degradation enzymes

The surface hydrophobicity of the chlorophyll-degrading enzyme is illustrated. White and red indicate the hydrophilic and hydrophobic regions, respectively. Docked chlorophyll derivatives are illustrated in green. AbSGR-h and 7-hydroxymethyl chlorophyll *a* reductase (HCAR) are prepared based on their respective crystal structures. AtSGR1 was predicted from the AbSGR-h structure using Swiss-Model. Chlorophyll *b* reductase (NYC1) and pheophytinase (PPH) structures are obtained from AlphaFold Protein Structure Database.

Figure 6. Schematic model of Stay-Green (SGR) interaction with their substrates

Chlorophyll *a* binds to the chlorophyll-protein complex localized in the hydrophobic thylakoid membrane. The hydrophobic region of Mg-dechelatase (SGR) is indicated in red. This hydrophobic region allows plant Mg-dechelatase to access chlorophyll *a*, whereas the

bacterial Mg-dechelataase homolog may not be adequately hydrophobic to access chlorophyll a.

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Fig. 1

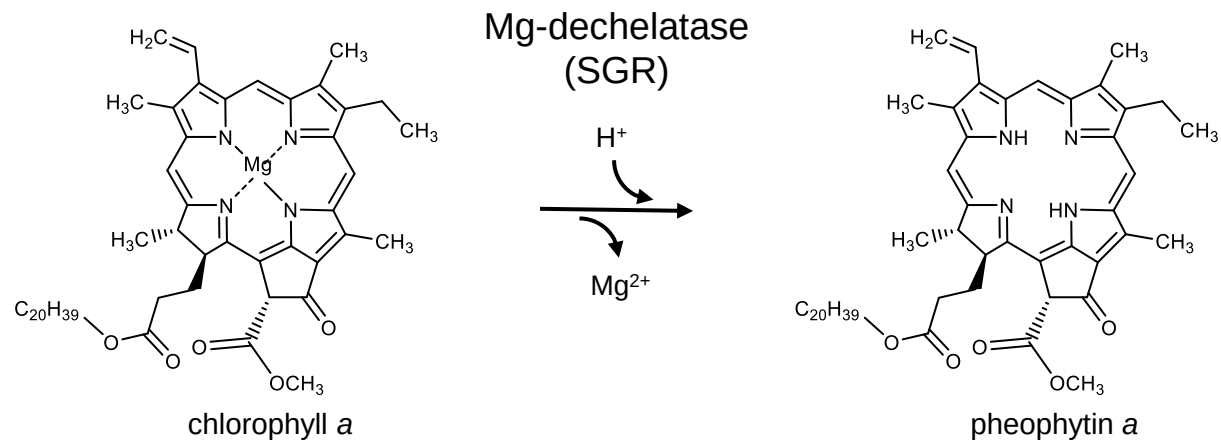
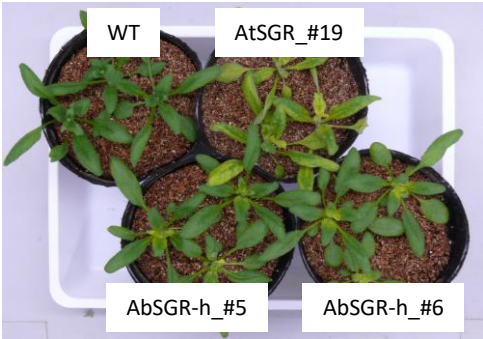
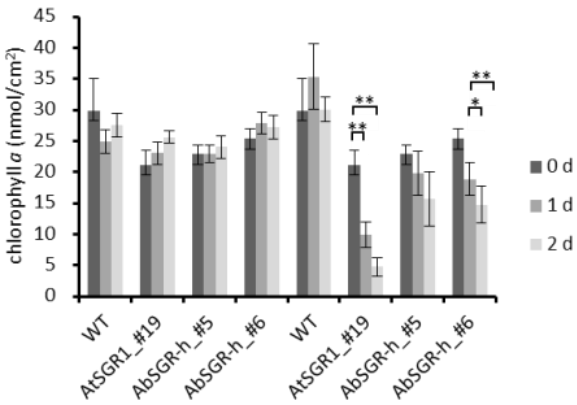


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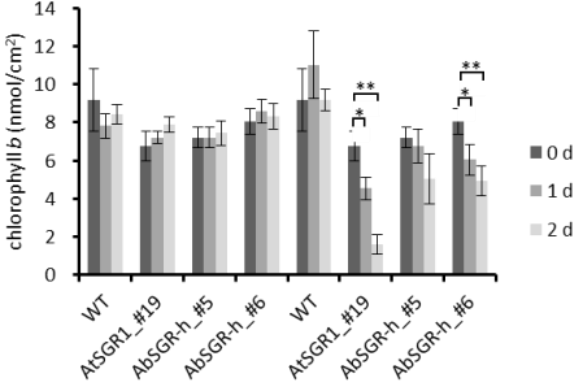
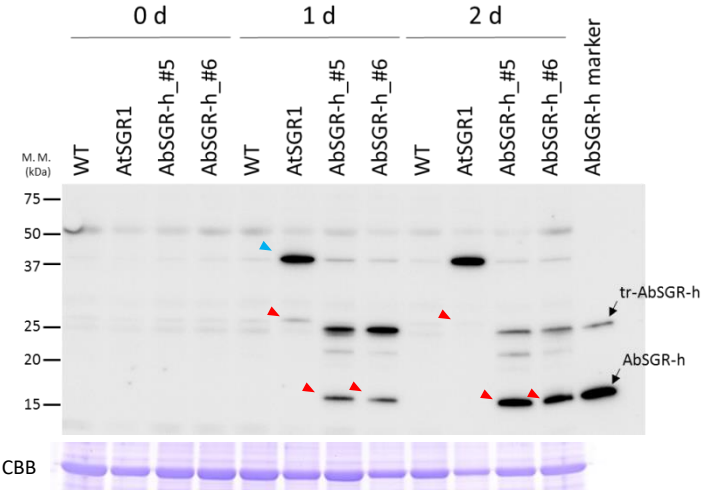
A



D



B



C

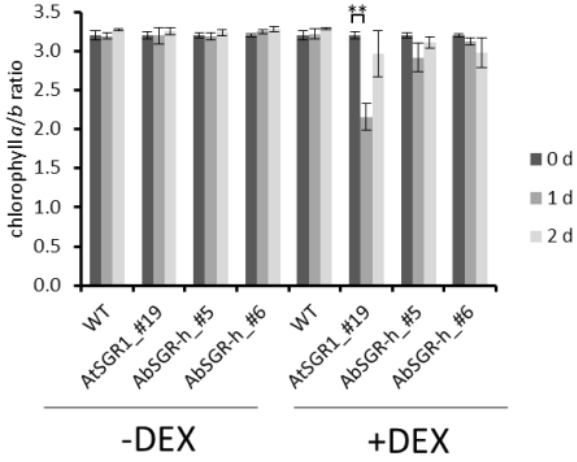
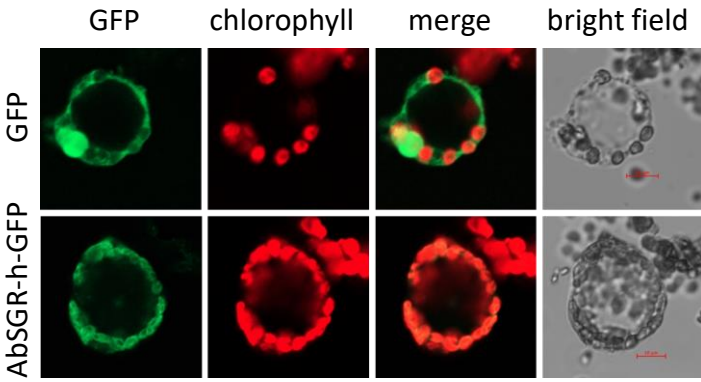
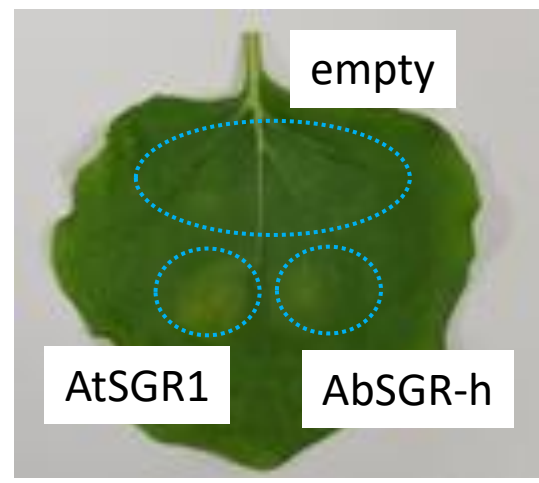
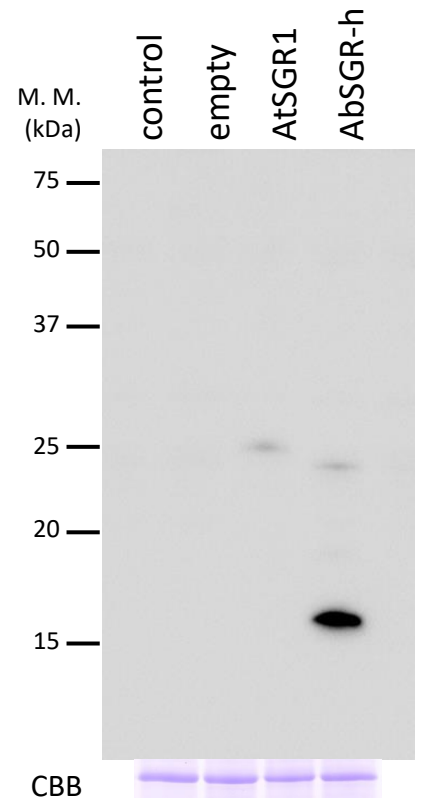


Fig. 3

A



B



C

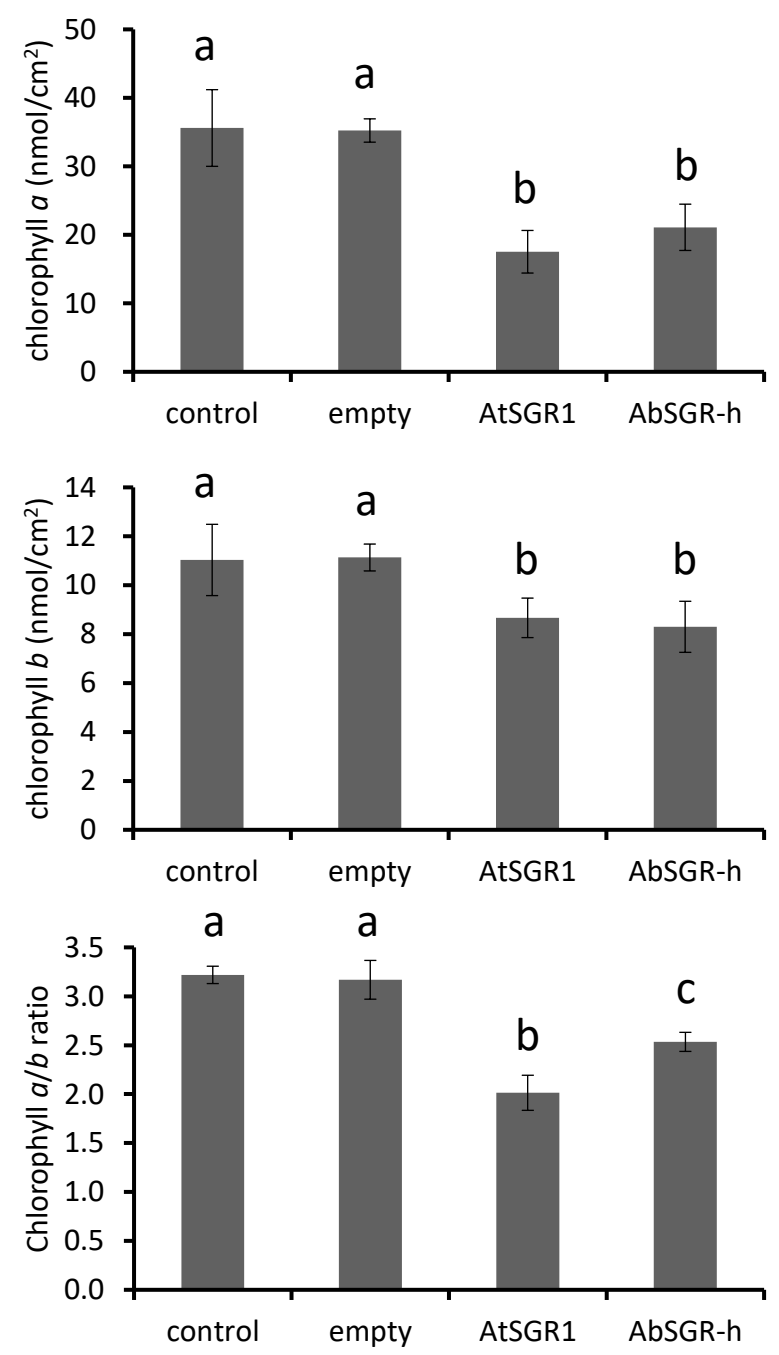


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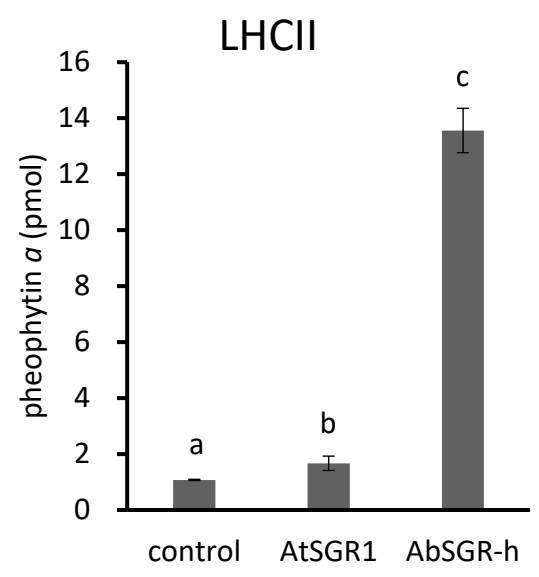
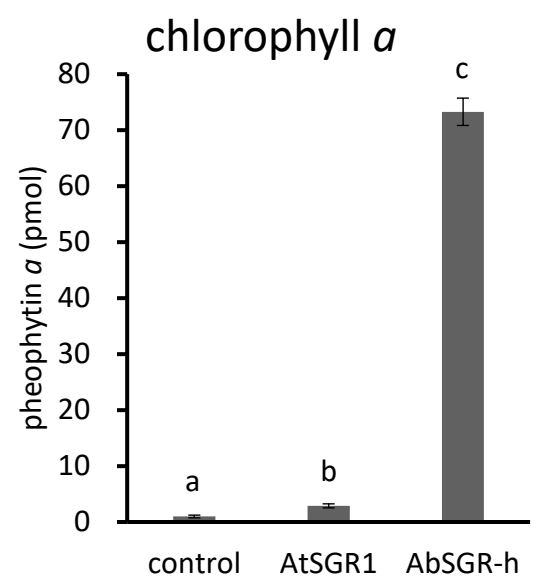


Fig. 5

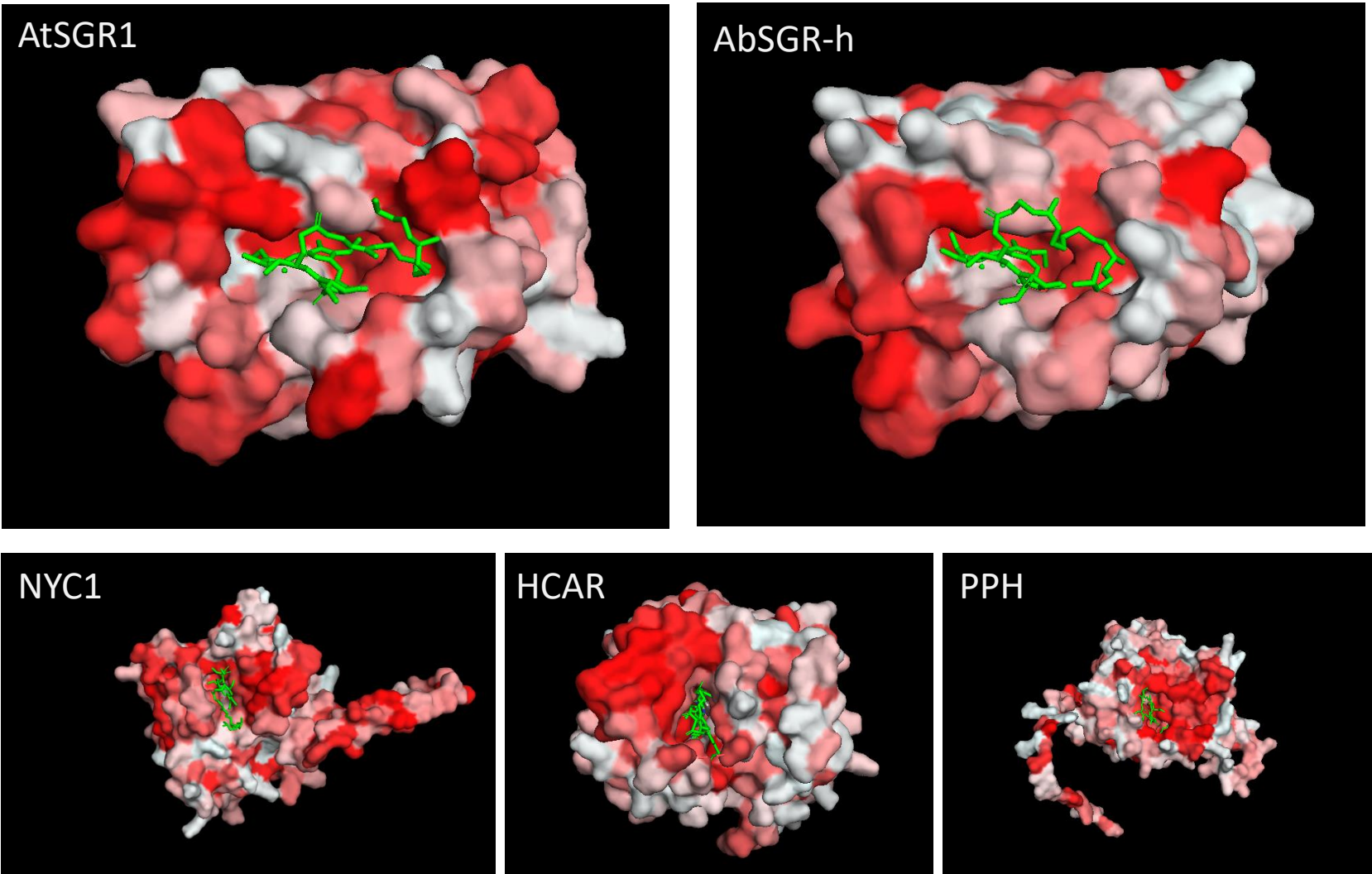


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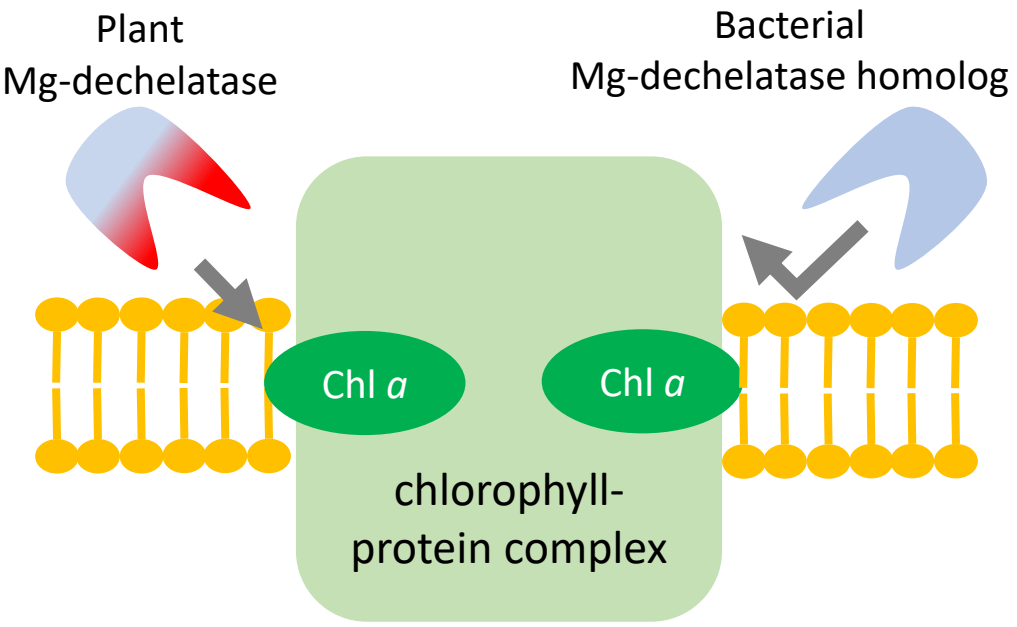


Table S1. Primer sets for PCR

|                                   |                                                                |
|-----------------------------------|----------------------------------------------------------------|
| AbSGR-h expression in Arabidopsis | 5'-3'                                                          |
| transit-F                         | GCAGGCTCCACCATGTGTAGTTTGTGCGCGATTAT                            |
| transit-R                         | TTTCAGATGATCCATAACGGGAACAATCGATTGGT                            |
| AbSGR-h-F                         | TCGATTGTTCCCGTTATGGATCATCTGAAACCGGA                            |
| AbSGR-h-R                         | AAGCTGGGTCTAGATCTACTTGTCGTCATCGTCTTTGTAGTCTTTGTAATCGCGCAGAATGC |
| Recombinant AbSGR-h               | 5'-3'                                                          |
| transit-F                         | AGGAGATATACATATGTGTAGTTTGTGCGCGAT                              |
| AbSGR-F                           | AAGGAGATATACATATGGATCATCTGAAACCGGAG                            |
| AbSGR-h-R                         | GGTGGTGGTGCTCGACTACTTGTCGTCATCGTCTT                            |
| AbSGR-h-GFP fusion                | 5'-3'                                                          |
| transit-F                         | TCTAGAGGATCCATGTGTAGTTTGTGCGCGAT                               |
| AbSGR-h-R                         | CCCTTGCTCACCATGGCCTTGTCGTCATCGTCTTTGT                          |

AtSGR1 (5'-3')

GCGCGTCTGTTCGGCCCGGCCATCTTCGAAAGCAGCAAGCTGAAGGTGCTGTTTCTGGGCGTGGACGAGA  
AAAAACACCCGAGCACGCTGCCACGCACGTACACGCTGACCCATAGCGATATCACCGCCAACTGACGCTG  
GCCATTAGCCAGAGCATCAACAACAGCCAGCTGCAAGGCTGGGCGAATCGTCTGTATCGCGATGAAGTTGT  
GGCGGAGTGGAAGAAGGTGAAAGGCAAGATGAGTCTGCACGTGCATTGCCATATCAGCGGCGGCCACTTT  
CTGCTGGACCTCTTCGCGAAGTTCCGCTACTTCATCTTCTGCAAGGAGCTGCCGGTGGTTCTGAAAGCGTTT  
GTGCATGGCGATGGCAATCTGCTGAATAACTACCCGGAAGTCCAAGAAGCGCTGGTGTGGGTGTACTTCCA  
CAGCAATGTGAACGAGTTCAACAAAGTGGAGTGCTGGGGTCCACTGTGGGAAGCCGTTAGCCCGGATGGC  
CATAAACGGAAACGCTGCCGGAAGCCCGTTGCGCCGATGAATGCAGCTGCTGCTTCCCGACCGTGAGCA  
GCATCCCGTGGAGCCACAGTCTGAGCAACGAAGGCGTTAATGGCTACAGCGGTACCCAGACCGAAGGCAT  
CGCGACCCCGAACCCGGAGAAACTG

AbSGR-h (5'-3')

ATGGATCATCTGAAACCGGAGAACTGCAGACCCGCTTTCTGAATGGCAGTCAGAACGATGGTCCGCGTTA  
TCCGCGCTGCTATACCCTGACCCACAGCGACAGCACCGGTGAACTGTTTCTGACCATTGGCCCGAGCTACGA  
TTACGAGCAGATTAGCGGCTGGTACACCCGCTTTATGCGCGATGAAGTGCTGGCCGTGTGGGAGATGGATG  
AAGAAGACATGGCACTGCACGTGCATGTGCATGTGAGCGGTGGTCTGATTCTGGGCAGCGCCAAATGGCG  
CGATAAAATCTTCCGCCAGCATATGCCGCTGGTTCTGGAAGCCTTTCGTTATGGCGATCGCGAGCTGGTGAA  
GAAGTATCCGGAGATGGATCAGGCCCCGATTCTGGTTCATTTCCATGCCCCGAACCCGAAATTCGATCTGGT  
GGAAACCTGGGGCATTCTGCGCGATTACAAA

Figure S1. Nucleotide sequences of AtSGR1 and AbSGR-h

AtSGR1 and AbSGR-h nucleotides were artificially designed to be optimized for expression in *E. coli*.

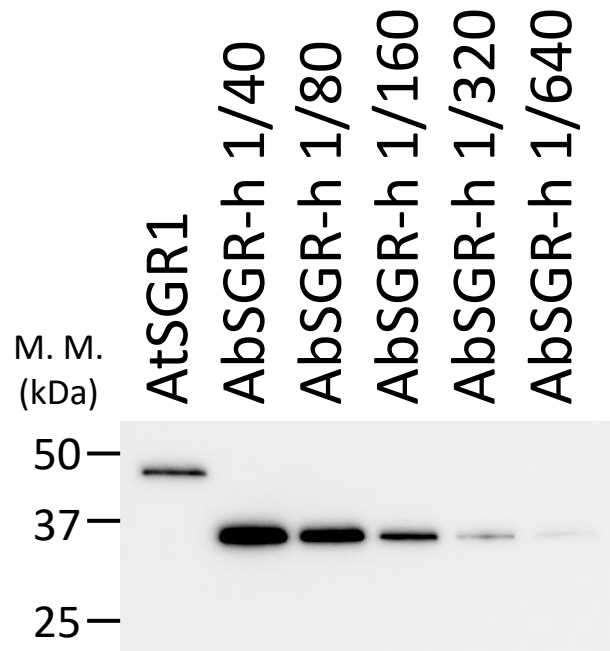


Figure S2. Normalization of AtSGR1 and AbSGR-h protein levels  
Protein concentrations of AtSGR1 and AbSGR-h were examined by immunoblotting against histidine tag.