Title

Degradation of the photosystem II core complex is independent of chlorophyll degradation mediated by Stay-Green Mg^{2+} dechelatase in *Arabidopsis*

Author names and affiliations

Ying Chen [1], Wataru Yamori[2], Ayumi Tanaka[1], Ryouichi Tanaka[1], Hisashi Ito[1]

[1]Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan

[2]Institute for Sustainable Agro-Ecosystem Services, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

Corresponding author

Hisashi Ito

Institute of Low Temperature Science, Hokkaido University, N19 W8, Sapporo 060-0819, Japan

Tel; +81-11-706-5493, Fax; +81-11-706-5493, E-mail; ito98@lowtem.hokudai.ac.jp

Keywords

chlorophyll degradation; photosystem degradation; senescence; Stay-Green.

Abbreviations

CBB (coomassie brilliant blue), Chl (chlorophyll), ETR (electron transport rate), LHCII (peripheral antenna complex of photosystem II), PS (photosystem), SGR (Stay-Green), SGRL (SGR-Like).

Author contributions

H.I. and A.T. conceptualized the original idea and planned each experiment. Y.C., W.Y., and H. I. executed the experiments. Y.C., A.T., R.T., and H.I. prepared the manuscript. All authors read and approved the manuscript.

Funding

This work was supported by the Japan Society for the Promotion of Science, KAKENHI [16H06556, 16H06552 and 18H02185 to W.Y.; 19K06700 to A.T.; 16H06554 to R.T.; and 17K07430 to H.I.].

Abstract

During leaf senescence, the degradation of photosystems and photosynthetic pigments proceeds in a coordinated manner, which would minimize the potential photodamage to cells. Both photosystem I and II are composed of core complexes and peripheral antenna complexes, with the former binding chlorophyll *a* and the latter binding chlorophyll *a* and *b*. Although the degradation of peripheral antenna complexes is initiated by chlorophyll degradation, it remains unclear whether the degradation of core complexes and chlorophyll is coordinated. In this study, we examined the degradation of peripheral antenna and core complexes in the Arabidopsis *sgr1/sgr2/sgrl* triple mutant, lacking all the isoforms of chlorophyll *a*:Mg^{2+} dechelatase. In this mutant, the degradation of peripheral antenna complexes and photosystem I core complexes was substantially retarded, but the core complexes of photosystem II were rapidly degraded during leaf senescence. On the contrary, the photosynthetic activity declined at a similar rate as in the wild type plants. These results suggest that the degradation of photosystem II core complexes is regulated independently of the major chlorophyll degradation pathway mediated by the dechelatase. The study should contribute to the understanding of the complex molecular mechanisms underlying the degradation of photosystems, which is an essential step during leaf senescence.

1. Introduction

A typical sign of the progress of leaf senescence is the yellowing of green leaves caused by chlorophyll (Chl) degradation. Land plants have two types of Chl, namely Chl *a* and Chl *b*. Chl *a* is bound to both the core and peripheral antenna complexes of the photosystems (PS), whereas Chl *b* is bound only to the peripheral antenna complex [1]. The first step in Chl *a* degradation is the production of pheophytin *a* upon extraction of the central Mg^{2+} by an Mg^{2+} dechelatase, which is alternatively called the Stay-Green (SGR, also known as NYE1) protein [2]. The phytyl group of pheophytin *a* is then removed by pheophytinase to form pheophorbide *a* [3]. The macrocycle ring of pheophorbide *a* is subsequently opened upon oxidation by pheophorbide *a* oxygenase [4]. The product is further catabolized in vacuoles by several enzymes [5, 6]. When Chl *b* is degraded, it is converted to Chl *a* through two successive reduction steps. The first step, catalyzed by Chl *b* reductase, results in the production of 7-hydroxymethyl Chl *a* [7]. 7-Hydroxymethyl Chl *a* reductase catalyzes the second step of the reduction to produce Chl *a* [8]. Chl *b*-containing cyanobacteria are also capable of converting Chl *b* to Chl *a* [9].

Mutations in the genes involved in the Chl degradation pathway impair Chl degradation, resulting in the delay in yellowing of leaves during senescence [6]. Deficiency in SGR and Chl *b* reductase results in a remarkable stay-green phenotype, indicating that these enzymes catalyze the reaction in the committed step of Chl degradation. Mutants in pheophorbide *a* oxygenase and 7-hydroxymethyl Chl *a* reductase do not show the significant stay-green phenotype under light/dark growth cycle conditions; however, green leaves are maintained in these mutants when senescence is induced under dark conditions.

In plants, the SGR proteins are classified into two subgroups: one group is called SGR, and the other is called SGR-Like (SGRL). Proteins in both the groups show Mg^{2+} dechelatase activity *in vitro* [2]. SGR is usually expressed during leaf senescence, whereas SGRL is expressed constitutively [6]. Arabidopsis has two isoforms of SGR (SGR1 and SGR2) and one of SGRL. Although SGR1 and SGR2 are responsible for almost all the Mg^{2+} dechelatase activity during leaf senescence, a possible contribution of SGRL cannot be disregarded.

During senescence, the degradation of PSI and II is usually coordinated with Chl degradation. Two hypotheses for the degradation of Chl–protein complexes have been proposed [10]. According to one, a protease first digests the protein moiety of Chl–protein complexes and the released Chl molecules are degraded thereafter. The other hypothesis is that Chl catabolic enzymes degrade Chl molecules bound to proteins and Chl-depleted proteins are immediately digested by some proteases. A study on the Chl *b* reductase-deficient mutant showed that light harvesting Chl *a*/*b*-protein complexes (peripheral antenna complexes of both the photosystems) are degraded according to the latter hypothesis [7, 11, 12]. Chl *b* reductase-deficient mutants of rice and Arabidopsis cannot degrade Chl *b* and these mutants retain both Chl *b* and Chl *a*/*b*–protein complexes, whereas, Chl *a*–protein complexes are degraded. Chl *b* may protect the apoprotein of Chl–protein complexes from the protease action. This phenomenon is similar to the observation that protein modification can prevent proteins from the action of proteases. For example, N-glycosylation of asparagine residues blocks the activity of asparaginyl endopeptidase [13]. The protease might not be able to access the cleavage site of the glycosylated proteins. However, it is not clear whether and how the degradation of the core complexes (Chl *a*–protein complexes) is coordinated with Chl breakdown. To answer this question, the *sgr* mutant, which cannot degrade Chl *a*, is useful. In a study on the degradation of photosystems in the Arabidopsis *sgr1/sgr2* double mutant, peripheral antenna complexes were found to be stable during senescence [14]. In this study, although Chl degradation was assumed to be blocked by the knockout of SGR1 and SGR2, the remaining SGRL activity could possibly have contributed to the phenotype of the mutant.

In this study, we analyzed the *sgr* triple mutant of Arabidopsis, which lacks all the three isoforms of SGR, and found that degradation of the peripheral antenna complexes and the core complexes of PSI was substantially delayed in the mutant, whereas the degradation of PSII core complexes proceeded without any significant delay in the mutant. The results show that degradation of the PSII core complexes is regulated differently from that of other Chl–protein complexes.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana (Columbia ecotype) was used for the experiments. A triple mutant of *SGR* was obtained by crossing mutants lacking *SGR1* [15] (a kind gift from Prof. B. Kuai, Fudan University), *SGR2* (SALK_003830C), and *SGRL* (SALK_084849), as previously reported [16]. Plants were

grown under 12 h light (100 μmol photons m^{-2} s^{-1})/12 h dark conditions at 24 °C. For dark-induced senescence, detached leaves were transferred to 24-well tissue culture plates, with water filled in the wells on the outermost side of the plates for maintaining humidity. The plates were sealed and covered with aluminum foil.

2.2. Chl and protein analysis

The 8th to 12th rosette leaves, counting from the bottom, were harvested for analysis. These leaves were almost fully expanded around the 5th week. Chl levels were measured with SPAD-502Plus (Konica Minolta). Leaf disks (0.25 cm^2) were frozen in liquid nitrogen and disrupted by shaking with zirconia beads. Protein was extracted by homogenization of the leaf disks in 250 μL of protein extraction buffer (125 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 5% [w/v] sucrose, 2.5% [w/w] 2-mercaptoethanol). After centrifugation (22,500 × *g*, 5 min), the supernatant was subjected to SDS-PAGE and the electrophoresed proteins were transferred onto a polyvinylidene difluoride membrane for immunoblot analysis as described previously [2]. For analysis of D1, 6 M of urea was used in the gel. All the target proteins were detected using primary antibodies from Agrisera, except for the antibody against CP1 [2]. The primary antibodies were diluted with an immunoreaction enhancer solution (Can Get Signal, Toyobo). Anti-rabbit IgG linked to horseradish peroxidase was used as the secondary antibody. Horseradish peroxidase activity was visualized using a Western Lightning Plus-ECL Chemiluminescence Detection Kit (PerkinElmer). Five microliter of the supernatant was used to detect PsaD, CP43, Lhca1, Lhca2, Lhca4, and Cyt b_6 and 0.5 μL was used to detect CP1 and D1. For coomassie brilliant blue (CBB) staining, 5 μL of the supernatant was used.

2.3. Transmission electron microscopy

Transmission electron microscopy was performed according to the method previously reported, with slight modifications [7]. HEPES buffer (60 mM HEPES-NaOH, pH 7.4, 200 mM NaCl, 4 mM $CaCl_2$) was used instead of 0.1 M cacodylate buffer. Briefly, leaves were soaked in a primary fixation buffer (2.5% glutaraldehyde in HEPES buffer). After rinsing three times in HEPES buffer, the leaves were fixed with a secondary fixation buffer (1% OsO_4 in HEPES buffer). The samples were dehydrated in a gradient series of ethanol and propylene oxide, and then embedded in an Epon resin mixture (Quetol 812, Nisshin EM). Ultrathin sections were stained with EM stainer (Nisshin EM) and 2% (w/v) lead citrate. Photographs were taken using a transmission electron microscope (H-7650, Hitachi).

2.4. Analysis of gas exchange, Chl fluorescence, and P700 measurements

Gas exchange, Chl fluorescence, and P700 redox state were measured simultaneously with a portable gas exchange system (GFS-3000, Walz) and a pulse amplitude-modulated fluorometer (Dual-PAM-100, Walz). A saturating pulse of light was applied to leaves that were dark-adapted for 30 min to obtain the maximum fluorescence and maximum oxidizable P700. Several photosynthetic parameters were measured at a CO_2 concentration of 400 μmol mol^{-1} and relative humidity of 60%–

70%; the cuvette temperature was kept 25 °C. The quantum yields of PSI (Y (I)) and PSII (Y (II)) were calculated as described previously [17]. The electron transport rates (ETR) through PSI and PSII were calculated as: ETR(I or II) = 0.5 × 0.84 × I × Y(I or II) , where 0.5 is the fraction of absorbed light allocated to PSI or PSII, 0.84 is the leaf absorption, and I is the incident irradiance [17].

3. Results

3.1. Degradation of Chl and PS proteins during natural senescence

To examine the effects of the deletion of all three SGR genes on Chl degradation during senescence, the wild type and *sgr1*/*sgr2*/*sgrl* triple mutant (hereafter, referred to as the *sgr* mutant) plants were grown under 12 h light/12 h dark conditions (Fig. 1A). The wild type and *sgr* mutant plants showed similar increases in Chl levels between the 3rd and the 5th week of growth. The leaves were still growing and becoming thicker during this stage. From the 6th to the 9th week of growth, the Chl content decreased linearly in the wild type. In contrast, the Chl content in the *sgr* mutant was maintained until the 9th week. Suppression of Chl degradation in the mutant indicates that SGR has a major role in Chl degradation in Arabidopsis.

We examined the protein levels by SDS-PAGE and CBB staining every 2 weeks from the 3rd to the 9th week of growth (Fig. 1B). The decrease in Rubisco is a good indicator of senescence because this protein is the largest source of nitrogen, which is relocalized to the sink organs during senescence [18]. The levels of the large subunit of Rubisco were similar from the 3rd to the 7th week, and were dramatically decreased at the 9th week both in the wild type and *sgr* mutant plants. The synchronized degradation of Rubisco in the wild type and *sgr* mutant plants suggests that the entire senescence program proceeds in a similar manner in these plants except for Chl degradation. The changes in the peripheral antenna complex of PSII (LHCII) were examined by CBB staining. In the wild type plants, LHCII almost disappeared at the 9th week as did Rubisco. However, LHCII bands were observed in the *sgr* mutant even at the 9th week. The presence of LHCII might not be because of the delay in senescence but because of the inhibition of Chl degradation. Stability of LHCII during senescence in the *sgr* mutant indicates that degradation of Chl *a* is indispensable for LHCII degradation. An alternate explanation is that Chl *b* reductase activity was kept low in the mutant because Chl *b* reductase induces degradation of the peripheral antenna complex [7]. Co-regulation of SGR and Chl *b* reductase remains to be studied.

We analyzed the levels of individual protein constituents of the photosystems by immunoblotting (Fig. 1C). At the 3rd and 5th week of growth, the levels of all the proteins were almost identical between the wild type and *sgr* mutant plants, suggesting that SGR has no effect on the accumulation of the components of the photosystems. The intensity of the protein bands on the gel was decreased at the 7th week and the bands almost disappeared at the 9th week in the wild type. In the *sgr* mutant, band intensities of these proteins did not change significantly at the 7th week. The CP1

(PsaA and PsaB complex) and PsaD bands were still observed at the 9th week, but their intensity was decreased slightly. The CP43 and D1 bands almost disappeared in the *sgr* mutant. These results suggest that PSI core complexes are resistant to degradation when they bind Chl, whereas PSII core complexes are sensitive to protease even when Chl degradation by SGR is impaired. The levels of Lhca1 and Lhcb2 did not decrease in the *sgr* mutant at the 9th week. The intensity of Lhcb4 (the minor antenna complex of PSII) band was decreased but it was still observed at the 9th week. Cyt b_6 is a representative protein that is degraded during senescence [19]. The Cyt b_6 band almost disappeared at the 9th week in the *sgr* mutant as well as in the wild type plant. This suggests that the wild type and *sgr* mutant plants were at a similar stage of senescence, consistent with the pattern observed for Rubisco (Fig. 1B).

3.2. Structures of cells and chloroplasts at the senescent stage

Degradation of intracellular organelles is a significant feature of a senescent cell. The intracellular structures in the wild type and *sgr* mutant plants can be assumed to be different because Chl levels in the senescent leaves were different (Fig. 1A). We observed the chloroplast structure by transmission electron microscopy (Fig. 2). Cells from 5-week-old wild type and *sgr* mutant leaves showed well-developed structure with mature chloroplasts. Well-organized thylakoid membranes and accumulation of starch granules was observed in the chloroplasts. No difference in the chloroplast structure was observed between the wild type and *sgr* mutant plants. At the 9th week, intracellular organelles were degraded and a swollen vacuole was seen in the wild type. We could not find traces of chloroplasts at this stage in the wild type. In the *sgr* mutant, round chloroplasts were observed in cells at the 9th week. Although thylakoid membranes were present in these chloroplasts, their overall structure was partly disorganized. Plastoglobules were visible in these chloroplasts. These observations suggest that when Chl degradation is suppressed, chloroplasts are not degraded properly. In a previous report on a Chl *b* reductase deficient mutant, the peripheral antenna complex was not degraded and a highly stacked grana structure was observed in senescent leaves [7]. The peripheral antenna complex was intact in the *sgr* mutant, but the grana stack was not seen. In addition to the peripheral antenna complex, the PSI core complex was present in the *sgr* mutant at the 9th week. This PSI core complex may interfere with grana stacking because of the large protrusion of the PSI structure at the stromal side.

3.3. Degradation of Chl and photosystem proteins during dark-induced senescence

Immunoblot analysis showed that the PSII core complex was degraded in the *sgr* mutant, which contradicts with the notion that Chl degradation is indispensable for the degradation of the Chl–protein complexes. A possible mechanism for the degradation of the PSII core complexes in the *sgr* mutant is photodamage, because the mutant plants were grown under light–dark cycle conditions (Fig. 1). To determine if the degradation of these proteins was caused by photodamage, senescence was induced in dark-adapted leaves to obviate photodamage. During the dark incubation, the Chl content was decreased sharply on the 4th day and then to almost 0 on the 7th day in the wild type (Fig. 3A). In the *sgr* mutant, almost half of the Chl content was retained after 7 days of incubation in the dark. The overall protein profiles analyzed by CBB staining and immunoblotting were similar

to those observed under natural senescence (Fig. 3B and C). Rubisco and Cyt b_6 were degraded in both the wild type and *sgr* mutant plants after dark incubation, suggesting that senescence proceeded at similar rates in both the plants. The degradation of Chl–protein complexes was suppressed in the *sgr* mutant after dark incubation except for the core complex of PSII. CP43 and D1 were retained for 4 days and disappeared after 7 days of dark incubation. These observations suggest that the degradation of the core complex of PSII is independent of photodamage. The chloroplast structure was also observed by transmission electron microscopy. The thylakoid membrane was retained after dark incubation of the *sgr* mutant (Fig. 2). The remaining Chl–protein complexes might have stabilized the thylakoid membrane in the *sgr* mutant.

3.4. The photosynthetic activity at the senescent stage

Because the stay-green mutants retain Chl at the senescent stage, we investigated the photosynthetic activity in these plants. Some stay-green mutants are photosynthetically functional whereas others are not [20]. The responses of CO_2 assimilation rate, stomatal conductance, and electron transport rate through PSII (ETRII) to light were determined at the senescence stage in the wild type and *sgr* mutant plants (Fig. S1). These parameters varied considerably in response to light depending on the age. However, these responses were similar in the wild type and *sgr* mutant plants. The CO_2 assimilation rates were measured at an illumination of 600 µmol photons m^{-2} s^{-1} (Fig. 4A). The wild type and *sgr* mutant plants showed a similar decrease in the CO_2 assimilation rate. At the 6th week, the CO_2 assimilation rates were slightly decreased compared to those at the 5th week. From the 6th to the 9th week, the CO_2 assimilation rates decreased linearly to almost zero. Although high levels of Chl were retained in the *sgr* mutant (Fig. 1A), the photosynthetic activity was lost at the late stage of senescence. The ETRII values were measured at an illumination of 600 µmol photons m^{-2} s^{-1} at the 5th and 8th week (Fig. 4B). The decrease in ETRII in the wild type and *sgr* mutant plants was similar to that at the 8th week. The electron transport rate through PSI (ETRI) values at 600 µmol photons m^{-2} s^{-1} showed different profiles. At the 5th week, the values in the wild type and *sgr* mutant plants were similar. At the 8th week, the *sgr* mutant showed a higher ETRI than the wild type plant. These observations suggest that although the *sgr* mutant has higher ETRI, ETRII decreases, resulting in a decrease in the net photosynthesis rate. These results are consistent with those of the immunoblot analysis, which shows that the core complex of PSII was degraded at the late stage of senescence (Fig. 1C).

4. Discussion

4.1. Effect of Chl degradation on photosystem degradation

Studies on the biogenesis of photosystems during the etioplast-to-chloroplast transition and those on the Chl synthesis mutants have shown that photosystem subunits need to bind Chl for their stable accumulation. Angiosperms cannot synthesize Chl, and therefore do not accumulate photosystems, in the dark. Nevertheless, the genes encoding the photosystem subunits are transcribed and translated under dark conditions in these plants [21]. It is likely that binding of Chl to the

apoproteins prevents the degradation of these proteins. Likewise, Chl *b*-less mutants do not accumulate LHCII even though the mRNA of LHCII apoprotein is transcribed and translated [22], indicating that Chl prevents the degradation of Chl-binding proteins. On the contrary, Chl degradation causes degradation of the Chl-binding proteins. This has been shown in studies on conditional induction of Chl degradation and those on mutants that are unable to degrade Chl [2, 23]. In this study, the effect of Chl degradation on the degradation of the photosystems was analyzed in the *sgr1/sgr2/sgrl* triple mutant. The results showed that the core complex of PSII was degraded even when Chl degradation by SGR was suppressed. This indicates that protease can readily digest the PSII core proteins even when Chl molecules are bound to these proteins. Another possibility is that Chl molecules are somehow pulled out from the core PSII subunits before they are digested by proteases. Alternatively, another chlorophyll catabolic enzyme besides SGR, such as chlorophyll dephytylase 1 [24], attacks Chl and then the protein moiety could be digested by protease.

It has been reported that dark treatment of SGR1-deficient Arabidopsis mutant leads to the degradation of D1 and D2 [25]. The PSII core complex in the rice *sgr* mutant was reported to be degraded after dark treatment [26]. D1 also disappeared in a pea mutant under the dark conditions [27]. These observations are consistent with our study on dark-induced senescence in the *sgr* mutant. The mutants used in these previous studies have SGRL. We used a mutant with no SGR genes. Therefore, degradation of the PSII core complex observed in this study definitively demonstrates that Chl degradation by SGR is not necessary for PSII degradation. The *Chlamydomonas sgr* mutant degraded Chl under nitrogen limiting conditions, suggesting the existence of an SGR-independent Chl degradation pathway in *Chlamydomonas* [16]. It is not clear if Chl bound to PSII is degraded by some unidentified process in Arabidopsis. Selective degradation of chloroplast materials delivered to vacuoles might also be possible in the degradation of photosystems [28].

In this study, we observed that the susceptibility of PSII core complexes to degradation by protease is different from that of other Chl–protein complexes. Identification of proteases involved in the degradation of PSII core complexes and other Chl–protein complexes is important to understand the process of degradation of photosystems. Proteases belonging to the FtsH and Deg families are considered to be involved in the degradation of Chl–protein complexes [29]. However, the protease that degrades the Chl–protein complexes during senescence has not been identified conclusively [30]. One of the reasons hampering the identification of proteases could be that the proteases have overlapping specificities toward their substrates and therefore a mutation in a specific protease may not result in the retention of a specific protein. On the contrary, D1 degradation during photodamage has been extensively studied and FtsH has been identified to be responsible for this degradation [31]. Nevertheless, the mechanism of photodamage-independent degradation of D1 during senescence has not been elucidated. Defect in THF1/Psb29 results in a stay-green phenotype and the retention of PSII during dark incubation in the mutants [25, 26]. THF1/Psb29 is associated with the FtsH protease complex [32]. These observations suggest that FtsH is also involved in PSII degradation independently of the photodamage at the senescence stage. The reason why only PSII core complex undergoes Chl-independent degradation and the mechanism by which PSII core complex is degraded needs to be clarified in the future.

4.2. Balance in the degradation of the photosystem components

Chl–protein complexes are large sources of nitrogen in leaves [33, 34]. Degradation of a Chl–protein complex and relocalization of the constituent nitrogen facilitate plant growth and seed generation [35]. Therefore, the cell needs to stay metabolically active until it finishes salvaging the nitrogen. It would be reasonable that PSI remains active longer than PSII because cells can produce ATP by the cyclic electron transfer around PSI. In contrast, PSII would be useless if PSI is absent because PSII requires the presence of PSI for linear electron transfer. Thus, cells would benefit from the labile nature of PSII compared with PSI. However, previous studies have shown that the balance of PSI and PSII degradation depends on plant species [36]. A preferential loss of PSI core complexes was observed in wild type barley and rice during natural senescence [37, 38]. The earlier onset of PSI degradation relative to that of PSII was also observed in maple and oak [39]. In contrast, PSI was more stable during senescence in tobacco [40] and Arabidopsis [41]. This study shows that when Chl degradation is suppressed, degradation of PSII core complexes is not retarded (Fig. 4B). This indicates that PSII is less stable than PSI. Uncoupling of the degradation of PSII and Chl might be advantageous from the perspective of preventing photodamage when Chl degradation is disturbed under conditions, such as low temperatures [42]. As a result of the degradation of the PSII core complex, free LHCII, which is not energetically connected to PSII, accumulates, but it is less likely to cause photodamage because of its ability to dissipate the absorbed light energy as heat [43]. Although degradation of the Cytb_6/*f* complex causes photodamage to PSII because this complex is involved in a rate-limiting step in the electron transfer system [44], its degradation may be advantageous in reducing the irreversible damage caused to PSI by over-reduction.

4.3. Relationship between the inhibition of Chl degradation and maintenance of photosynthetic activity

In general, mutants that maintain green color during senescence are expected to have the potential for long-term photosynthesis [20]. However, the mutants impaired in Chl degradation are usually non-functionally stay-green [45] because almost all the processes of senescence, except for Chl degradation, proceed at rates similar to those in the wild type plants [23]. In this study, the *sgr* mutant lost its photosynthetic activity during senescence at the same rate as the wild type plants; this is consistent with the phenotype reported for other mutants impaired in Chl degradation. This may be because the levels of PSII, Cytb_6/*f* complex, and Rubisco decreased during senescence as in the wild type plants. Although Chl retention does not result in the retardation of senescence, Chl degradation can affect its progress. Jasmonate production was observed when Chl degradation was induced by temporal induction of SGR [46]. Jasmonate promotes senescence; therefore, Chl degradation can be supposed to affect the promotion of senescence through the action of jasmonate. Pheophorbide *a* is an intermediate molecule in the Chl degradation pathway. The mutant accumulating excess pheophorbide *a* showed an elevated level of jasmonate during dark-induced senescence, suggesting that pheophorbide *a* may regulate jasmonate signaling [47]. This observation also suggests that Chl degradation may affect the progress of senescence. Although the stay-green mutant defect in Chl degradation by SGR is a non-functional stay-green as described above, delayed expression of *SGR* led to a prolonged photosynthesis and an increase in biomass

[48]. This indicates that regulation of Chl degradation by SGR can indeed be used to maintain the photosynthetic function.

A number of mutants that maintain a green color owing to mutations other than those in the Chl degradation system are also known [23, 49]. For example, ethylene-insensitive mutants maintain their green color for a longer duration. This is because ethylene is involved in the expression of a gene in the Chl-degradation system, and therefore, these mutants do not induce the Chl-degradation system sufficiently. In these mutants, the degradation of all the Chl–protein complexes, including the core complex of PSII, is suppressed during senescence. These results suggest that the protease that degrades the core complex of PSII is induced by plant hormones. The degradation of photosystems involves cooperative action of Chl catabolic enzymes and proteases. These complicated reactions need to be examined through a comprehensive analysis of the metabolic network. Transcriptome [50] and proteome analyses of the chloroplast [51] during senescence provide an overall picture of the progress of senescence. However, each metabolic activity is controlled not only by transcription, but also by translation or at the other levels of regulatory mechanisms and the roles of such mechanisms need to be studied in detail. The present study contributes to the understanding of the complex molecular mechanisms underlying the degradation of photosystems, which is an essential step during leaf senescence.

Figure legends

Fig. 1 Changes in the levels of Chl and proteins during senescence

(A) Chl levels in the wild type and *sgr* mutant leaves of 3–9-week-old plants, expressed as SPAD values. The error bars represent the SD (n = 5). * $p < 0.05$, ** $p < 0.01$ (unpaired *t*-test). Proteins were extracted from leaf disks of the wild type and *sgr* mutant leaves. The results were normalized by leaf area. Proteins were stained with CBB. Rubisco large subunit (RbcL) and LHCII are indicated (B). Each protein was detected using a specific primary antibody (C).

Fig. 2 Transmission electron microscopy of the chloroplast structure

Wild type and *sgr* mutant leaves of 5- and 9-week-old plants, and 7-day dark incubated leaves of 5-week-old plants were observed by microscopy at low and high magnification. No trace of chloroplast was found in leaves from 9-week-old wild type plants. Scale bar = 100 μm at low magnification and 20 μm in high magnification.

Fig. 3 Changes in the levels of Chl and proteins during dark-induced senescence

(A) Leaves of 5-week-old wild type and *sgr* mutant plants were incubated in the dark to induce senescence. Chl levels after incubation in the dark for 4 and 7 days are shown as SPAD values. The error bars represent the SD (n = 3). ** $p < 0.01$ (unpaired *t*-test). Proteins were extracted from leaf disks of the wild type and *sgr* mutant leaves after dark incubation for 4 and 7 days. The results were

normalized by leaf area. Proteins were stained with CBB. Rubisco large subunit (RbcL) and LHCII are indicated (B). Each protein was detected using a specific primary antibody (C).

Fig. 4 Changes in the photosynthetic parameters during senescence

(A) CO_2 assimilation rates of 5–9-week-old leaves were measured at an illumination of 600 μmol photons m^{-2} s^{-1}. (B) ETRI and ETRII of 5- and 8-week-old leaves were measured at an illumination of 600 μmol photons m^{-2} s^{-1}. The error bars represent the SD (n = 4). ** $p < 0.01$ (unpaired *t*-test).

[1] N. Nelson, C.F. Yocum, Structure and function of photosystems I and II, Annu. Rev. Plant Biol., 57 (2006) 521-565.
[2] Y. Shimoda, H. Ito, A. Tanaka, Arabidopsis STAY-GREEN, Mendel's green cotyledon gene, encodes magnesium-dechelatase, Plant Cell, 28 (2016) 2147-2160.
[3] S. Schelbert, S. Aubry, B. Burla, B. Agne, F. Kessler, K. Krupinska, S. Hörtensteiner, Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in Arabidopsis, Plant Cell, 21 (2009) 767-785.
[4] A. Pruzinska, G. Tanner, I. Anders, M. Roca, S. Hörtensteiner, Chlorophyll breakdown: pheophorbide a oxygenase is a Rieske-type iron-sulfur protein, encoded by the accelerated cell death 1 gene, Proc. Natl. Acad. Sci. U. S. A., 100 (2003) 15259-15264.
[5] B. Kräutler, Breakdown of chlorophyll in higher plants—Phyllobilins as abundant, yet hardly visible signs of ripening, senescence, and cell death, Angew. Chem., Int. Ed., 55 (2016) 4882-4907.
[6] B. Kuai, J. Chen, S. Hörtensteiner, The biochemistry and molecular biology of chlorophyll breakdown, J. Exp. Bot., 69 (2018) 751-767.
[7] M. Kusaba, H. Ito, R. Morita, S. Iida, Y. Sato, M. Fujimoto, S. Kawasaki, R. Tanaka, H. Hirochika, M. Nishimura, A. Tanaka, Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence, Plant Cell, 19 (2007) 1362-1375.
[8] M. Meguro, H. Ito, A. Takabayashi, R. Tanaka, A. Tanaka, Identification of the 7-hydroxymethyl chlorophyll a reductase of the chlorophyll cycle in Arabidopsis, Plant Cell, 23 (2011) 3442-3453.
[9] H. Lim, A. Tanaka, R. Tanaka, H. Ito, In Vitro Enzymatic Activity Assays Implicate the Existence of the Chlorophyll Cycle in Chlorophyll b-Containing Cyanobacteria, Plant Cell Physiol., 60 (2019) 2672-2683.
[10] L. Dall'Osto, M. Bressan, R. Bassi, Biogenesis of light harvesting proteins, Biochim. Biophys. Acta, 1847 (2015) 861-871.
[11] Y. Horie, H. Ito, M. Kusaba, R. Tanaka, A. Tanaka, Participation of chlorophyll b reductase in the initial step of the degradation of light-harvesting chlorophyll a/b-protein complexes in Arabidopsis, J. Biol. Chem., 284 (2009) 17449-17456.
[12] Y. Sato, R. Morita, S. Katsuma, M. Nishimura, A. Tanaka, M. Kusaba, Two short-chain dehydrogenase/reductases, NON-YELLOW COLORING 1 and NYC1-LIKE, are required for chlorophyll b and light-harvesting complex II degradation during senescence in rice, Plant J., 57 (2009) 120-131.
[13] B. Manoury, E.W. Hewitt, N. Morrice, P.M. Dando, A.J. Barrett, C. Watts, An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation, Nature, 396 (1998) 695-699.

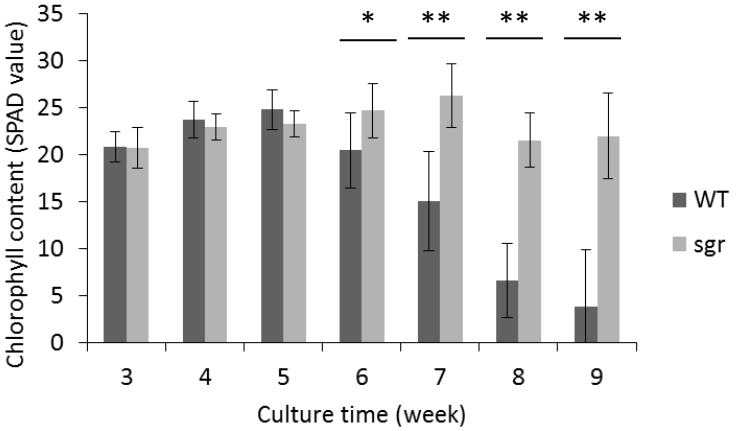
[14] S. Wu, Z. Li, L. Yang, Z. Xie, J. Chen, W. Zhang, T. Liu, S. Gao, J. Gao, Y. Zhu, J. Xin, G. Ren, B. Kuai, NON-YELLOWING2 (NYE2), a close paralog of NYE1, plays a positive role in chlorophyll degradation in Arabidopsis, Mol. Plant, 9 (2016) 624-627.
[15] G. Ren, K. An, Y. Liao, X. Zhou, Y. Cao, H. Zhao, X. Ge, B. Kuai, Identification of a Novel Chloroplast Protein AtNYE1 Regulating Chlorophyll Degradation during Leaf Senescence in Arabidopsis, Plant Physiol., 144 (2007) 1429-1441.
[16] Y. Chen, Y. Shimoda, M. Yokono, H. Ito, A. Tanaka, Mg-dechelatase is involved in the formation of photosystem II but not in chlorophyll degradation in Chlamydomonas reinhardtii, Plant J., 97 (2019) 1022-1031.
[17] C. Klughammer, U. Schreiber, An improved method, using saturating light pulses, for the determination of photosystem I quantum yield via $P700^{+}$-absorbance changes at 830 nm, Planta, 192 (1994) 261-268.
[18] Y. Suzuki, A. Makino, T. Mae, Changes in the turnover of Rubisco and levels of mRNAs of rbcL and rbcS in rice leaves from emergence to senescence, Plant Cell Environ., 24 (2001) 1353-1360.
[19] K. Humbeck, S. Quast, K. Krupinska, Functional and molecular changes in the photosynthetic apparatus during senescence of flag leaves from field-grown barley plants, Plant Cell Environ., 19 (1996) 337-344.
[20] H. Thomas, C.J. Howarth, Five ways to stay green, J. Exp. Bot., 51 (2000) 329-337.
[21] T. Armarego-Marriott, Ł. Kowalewska, A. Burgos, A. Fischer, W. Thiele, A. Erban, D. Strand, S. Kahlau, A. Hertle, J. Kopka, D. Walther, Z. Reich, M.A. Schöttler, R. Bock, Highly Resolved Systems Biology to Dissect the Etioplast-to-Chloroplast Transition in Tobacco Leaves, Plant Physiol., 180 (2019) 654-681.
[22] S. Nick, J. Meurer, J. Soll, E. Ankele, Nucleus-Encoded Light-Harvesting Chlorophyll a/b Proteins are Imported Normally into Chlorophyll b-Free Chloroplasts of Arabidopsis, Mol. Plant, 6 (2013) 860-871.
[23] M. Kusaba, A. Tanaka, R. Tanaka, Stay-green plants: what do they tell us about the molecular mechanism of leaf senescence, Photosynth. Res., 117 (2013) 221-234.
[24] Y.-P. Lin, Y.-y. Charng, Chlorophyll dephytylation in chlorophyll metabolism: a simple reaction catalyzed by various enzymes, Plant Sci., 302 (2021) 110682.
[25] W. Huang, Q. Chen, Y. Zhu, F. Hu, L. Zhang, Z. Ma, Z. He, J. Huang, Arabidopsis Thylakoid Formation 1 Is a Critical Regulator for Dynamics of PSII–LHCII Complexes in Leaf Senescence and Excess Light, Mol. Plant, 6 (2013) 1673-1691.
[26] H. Yamatani, Y. Sato, Y. Masuda, Y. Kato, R. Morita, K. Fukunaga, Y. Nagamura, M. Nishimura, W. Sakamoto, A. Tanaka, M. Kusaba, NYC4, the rice ortholog of Arabidopsis THF1, is involved in the degradation of chlorophyll – protein complexes during leaf senescence, Plant J., 74 (2013) 652-662.
[27] S. Aubry, J. Mani, S. Hörtensteiner, Stay-green protein, defective in Mendel's green cotyledon mutant, acts independent and upstream of pheophorbide a oxygenase in the chlorophyll catabolic pathway, Plant Mol. Biol., 67 (2008) 243-256.
[28] X. Zhuang, L. Jiang, Chloroplast Degradation: Multiple Routes Into the Vacuole, Front. Plant Sci., 10 (2019).
[29] K. Nishimura, Y. Kato, W. Sakamoto, Essentials of Proteolytic Machineries in Chloroplasts, Mol. Plant, 10 (2017) 4-19.
[30] K.J.v. Wijk, Protein Maturation and Proteolysis in Plant Plastids, Mitochondria, and Peroxisomes, Annu. Rev. Plant Biol., 66 (2015) 75-111.
[31] Y. Kato, W. Sakamoto, FtsH Protease in the Thylakoid Membrane: Physiological Functions and the Regulation of Protease Activity, Front. Plant Sci., 9 (2018).

[32] M. Bečková, J. Yu, V. Krynická, A. Kozlo, S. Shao, P. Koník, J. Komenda, J.W. Murray, P.J. Nixon, Structure of Psb29/Thf1 and its association with the FtsH protease complex involved in photosystem II repair in cyanobacteria, Philos. Trans. R Soc. Lond. B Biol. Sci., 372 (2017) 20160394.
[33] S. Hörtensteiner, U. Feller, Nitrogen metabolism and remobilization during senescence, J. Exp. Bot., 53 (2002) 927-937.
[34] A. Makino, H. Sakuma, E. Sudo, T. Mae, Differences between Maize and Rice in N-use Efficiency for Photosynthesis and Protein Allocation, Plant Cell Physiol., 44 (2003) 952-956.
[35] G. Xu, X. Fan, A.J. Miller, Plant Nitrogen Assimilation and Use Efficiency, Annu. Rev. Plant Biol., 63 (2012) 153-182.
[36] A. Krieger-Liszkay, K. Krupinska, G. Shimakawa, The impact of photosynthesis on initiation of leaf senescence, Physiol. Plant, 166 (2019) 148-164.
[37] I. Miersch, J. Heise, I. Zelmer, K. Humbeck, Differential Degradation of the Photosynthetic Apparatus During Leaf Senescence in Barley (Hordeum vulgare L.), Plant Biology, 2 (2000) 618-623.
[38] Y. Tang, X. Wen, C. Lu, Differential changes in degradation of chlorophyll–protein complexes of photosystem I and photosystem II during flag leaf senescence of rice, Plant Physiol. Biochem., 43 (2005) 193-201.
[39] A. Moy, S. Le, A. Verhoeven, Different strategies for photoprotection during autumn senescence in maple and oak, Physiol. Plant, 155 (2015) 205-216.
[40] M.A. Schöttler, W. Thiele, K. Belkius, S.V. Bergner, C. Flügel, G. Wittenberg, S. Agrawal, S. Stegemann, S. Ruf, R. Bock, The plastid-encoded PsaI subunit stabilizes photosystem I during leaf senescence in tobacco, J. Exp. Bot., 68 (2017) 1137-1155.
[41] K. Nath, B.-K. Phee, S. Jeong, S.Y. Lee, Y. Tateno, S.I. Allakhverdiev, C.-H. Lee, H.G. Nam, Age-dependent changes in the functions and compositions of photosynthetic complexes in the thylakoid membranes of Arabidopsis thaliana, Photosynth. Res., 117 (2013) 547-556.
[42] S. Hörtensteiner, B. Krautler, Chlorophyll breakdown in oilseed rape, Photosynth. Res., 64 (2000) 137-146.
[43] F. Saccon, V. Giovagnetti, M.K. Shukla, A.V. Ruban, Rapid regulation of photosynthetic light harvesting in the absence of minor antenna and reaction centre complexes, J. Exp. Bot., (2020).
[44] W. Yamori, S. Takahashi, A. Makino, G.D. Price, M.R. Badger, S. von Caemmerer, The Roles of ATP Synthase and the Cytochrome b6f Complexes in Limiting Chloroplast Electron Transport and Determining Photosynthetic Capacity, Plant Physiol., 155 (2011) 956-962.
[45] S. Hörtensteiner, Stay-green regulates chlorophyll and chlorophyll-binding protein degradation during senescence, Trends Plant Sci., 14 (2009) 155-162.
[46] K. Ono, M. Kimura, H. Matsuura, A. Tanaka, H. Ito, Jasmonate production through chlorophyll a degradation by Stay-Green in Arabidopsis thaliana, J. Plant Physiol., 238 (2019) 53-62.
[47] S. Aubry, N. Fankhauser, S. Ovinnikov, A. Pružinská, M. Stirnemann, K. Zienkiewicz, C. Herrfurth, I. Feussner, S. Hörtensteiner, Pheophorbide a May Regulate Jasmonate Signaling during Dark-Induced Senescence, Plant Physiol., 182 (2020) 776-791.
[48] D. Shin, S. Lee, T.-H. Kim, J.-H. Lee, J. Park, J. Lee, J.Y. Lee, L.-H. Cho, J.Y. Choi, W. Lee, J.-H. Park, D.-W. Lee, H. Ito, D.H. Kim, A. Tanaka, J.-H. Cho, Y.-C. Song, D. Hwang, M.D. Purugganan, J.-S. Jeon, G. An, H.G. Nam, Natural variations at the Stay-Green gene promoter control lifespan and yield in rice cultivars, Nat. Commun., 11 (2020) 2819.
[49] X. Zhu, J. Chen, K. Qiu, B. Kuai, Phytohormone and Light Regulation of Chlorophyll Degradation, Front. Plant Sci., 8 (2017).

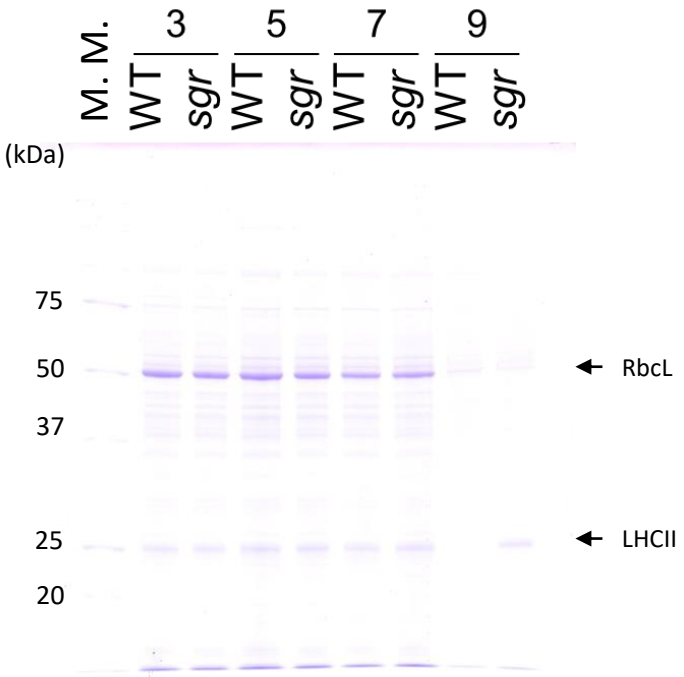
[50] E. Breeze, E. Harrison, S. McHattie, L. Hughes, R. Hickman, C. Hill, S. Kiddle, Y.-s. Kim, C.A. Penfold, D. Jenkins, C. Zhang, K. Morris, C. Jenner, S. Jackson, B. Thomas, A. Tabrett, R. Legaie, J.D. Moore, D.L. Wild, S. Ott, D. Rand, J. Beynon, K. Denby, A. Mead, V. Buchanan-Wollaston, High-Resolution Temporal Profiling of Transcripts during Arabidopsis Leaf Senescence Reveals a Distinct Chronology of Processes and Regulation, Plant Cell, 23 (2011) 873-894.
[51] E. Tamary, R. Nevo, L. Naveh, S. Levin-Zaidman, V. Kiss, A. Savidor, Y. Levin, Y. Eyal, Z. Reich, Z. Adam, Chlorophyll catabolism precedes changes in chloroplast structure and proteome during leaf senescence, Plant Direct, 3 (2019) e00127.

Fig. 1

A


Chlorophyll content (SPAD value)
35
30
25
20
15
10
5
0
*
**
**
**
3
4
5
6
7
8
9
Culture time (week)
WT
sgr

B


M. M.
3
5
7
9
WT
sgr
WT
sgr
WT
sgr
WT
sgr
(kDa)
75
50
37
25
20
RbcL
LHCII

C

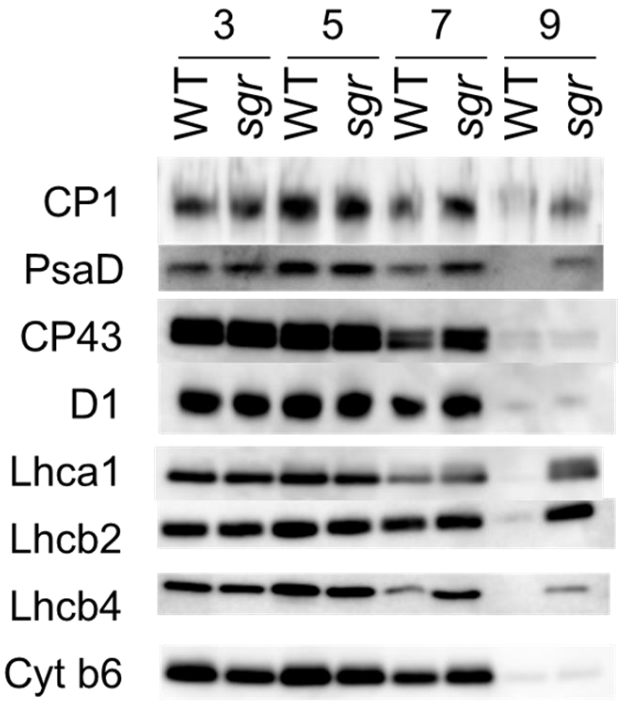

3
5
7
9
WT
sgr
WT
sgr
WT
sgr
WT
sgr
CP1
PsaD
CP43
D1
Lhca1
Lhcb2
Lhcb4
Cyt b6

Fig. 2

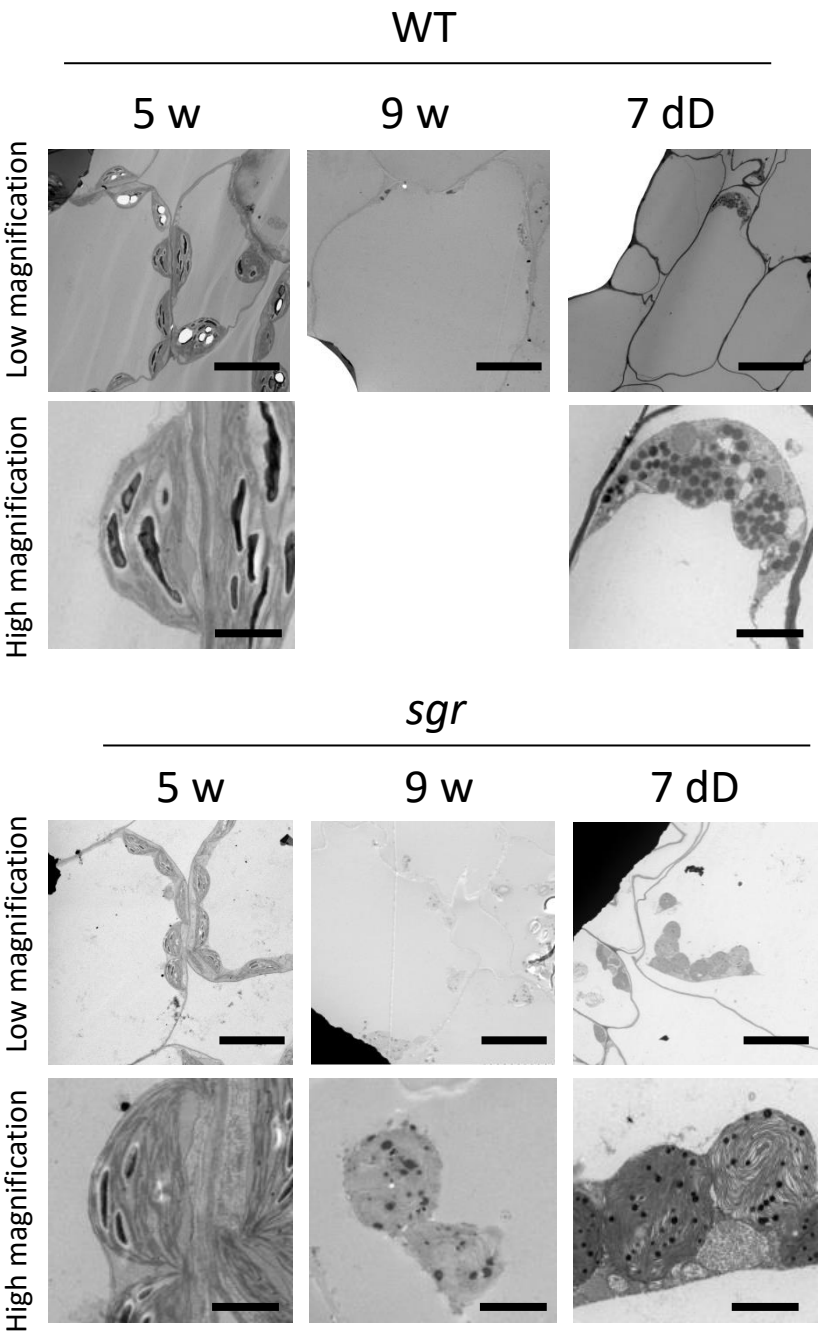
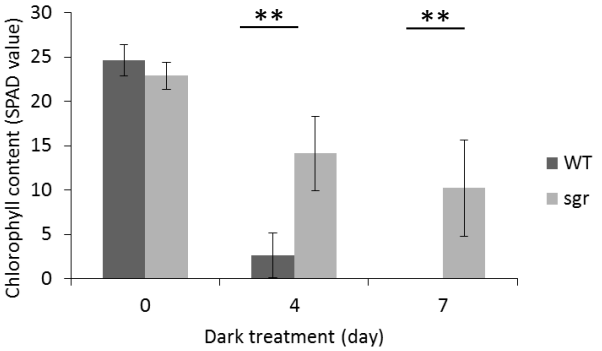
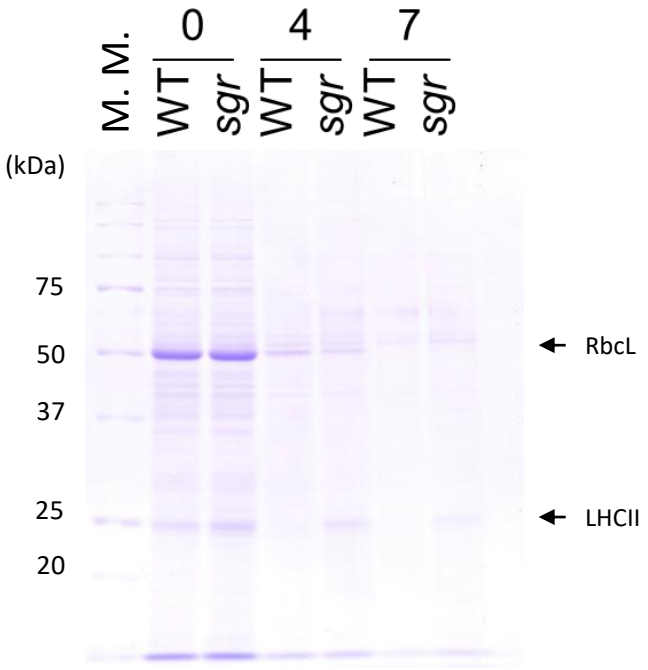

WT
5 w
9 w
7 dD
Low magnification
High magnification
sgr
5 w
9 w
7 dD
Low magnification
High magnification

Fig. 3

A


Chlorophyll content (SPAD value)
30
25
20
15
10
5
0
**
**
WT
sgr
0
4
7
Dark treatment (day)

B


M. M.
0
4
7
WT
sgr
WT
sgr
WT
sgr
(kDa)
75
50
37
25
20
RbcL
LHCII

C

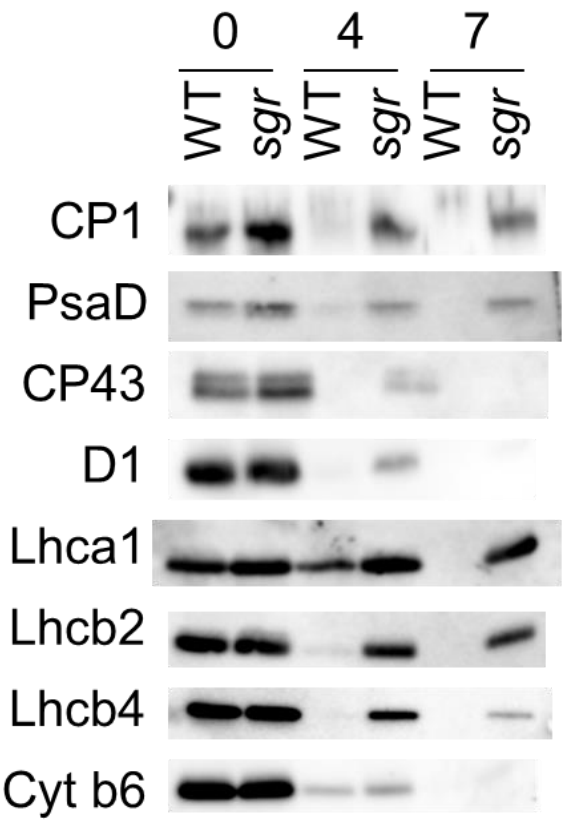
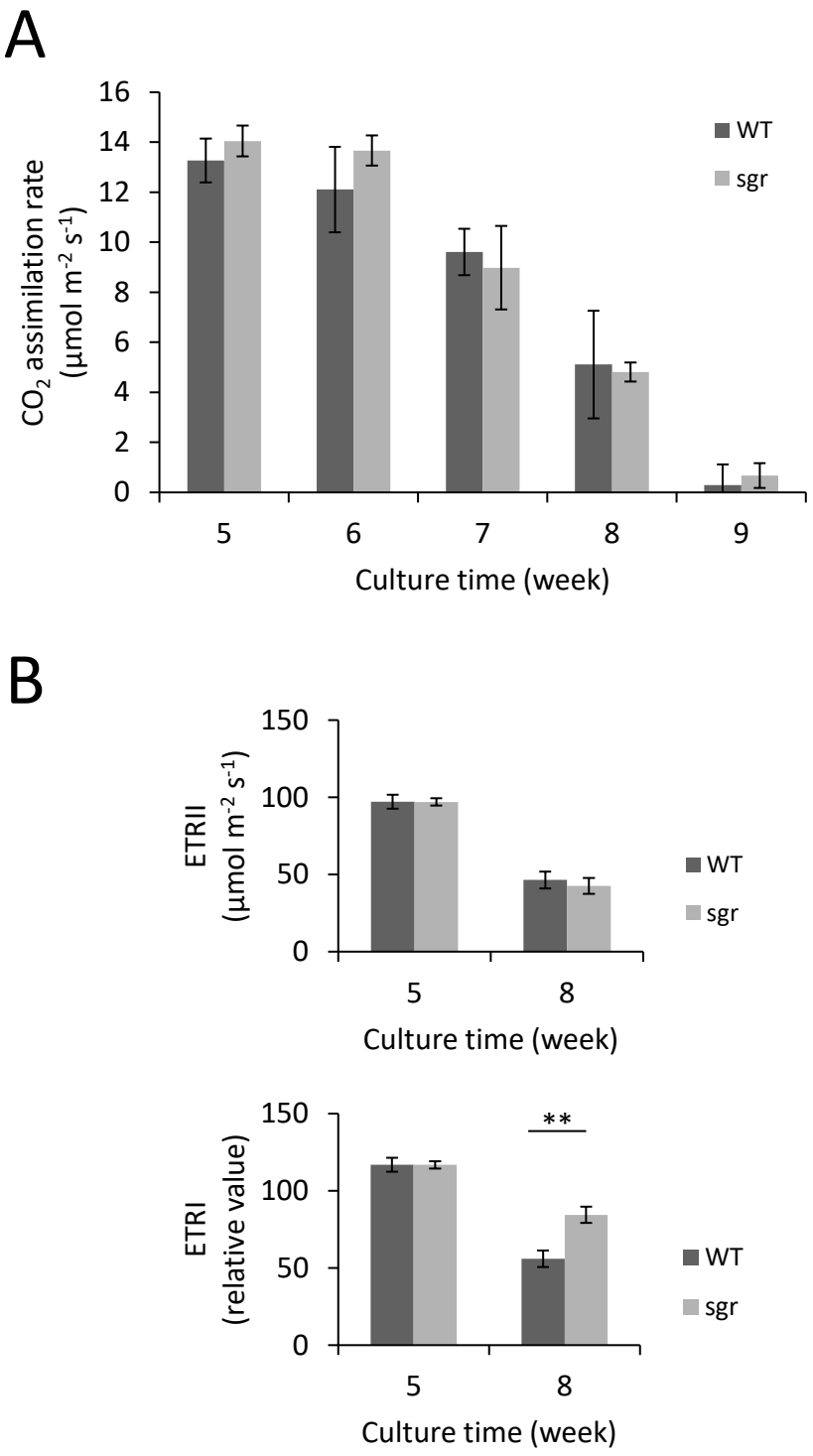
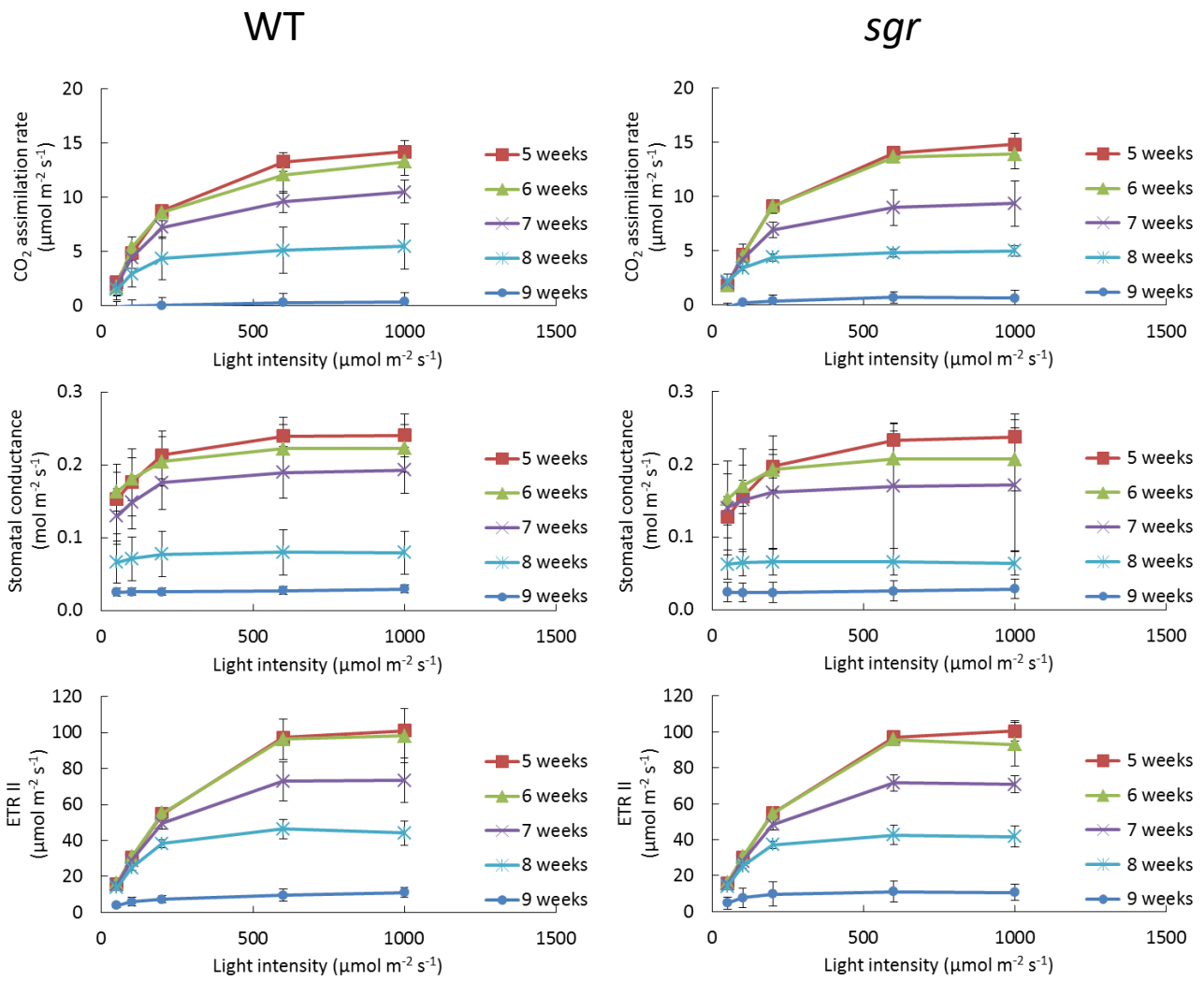

0
4
7
WT
sgr
WT
sgr
WT
sgr
CP1
PsaD
CP43
D1
Lhca1
Lhcb2
Lhcb4
Cyt b6

Fig. 4


A
16
14
12
10
8
6
4
2
0
CO_2 assimilation rate
(μmol m^{-2} s^{-1})
WT
sgr
5
6
7
8
9
Culture time (week)
B
150
100
50
0
ETRII
(μmol m^{-2} s^{-1})
WT
sgr
5
8
Culture time (week)
150
100
50
0
**
ETRI
(relative value)
WT
sgr
5
8
Culture time (week)



Supplementary Fig. S1 Response of CO_2 assimilation rates, stomatal conductance, and ETRII to light
Wild type and *sgr* mutant leaves of 5–9-week-old plants were analyzed. The error bars represent the SD (n = 4).