Synechocystis sp. PCC6803 における
光化学系 I の機能維持に関わる遺伝子の探索

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Exploration of genes that are involved in the functional maintenance of the Photosystem I in *Synechocystis* sp. PCC6803

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Abstract

The photosystem (PSI) I complex consisted of a lot of subunits has an important role to convert the chemical energy from the light energy. The biosynthesis of the PSI complex depends on complicated processes besides the coordinated expression of nuclear and chloroplast genes. In this study, I aimed to identify genes which are involved in the functional maintenance of the PSI complex by investigating *sll0933, slr0815 and slr0589* mutants which were expected to have abnormality in the downstream of the photosynthetic electron transport but not the subunits of PSI. I determined PSI/PSII ratio by chlorophyll fluorescence spectra determined at 77 K, Chl/Cell ratio by absorption spectra of intact cells and P-700 content by light-induced difference absorption change. The results indicate that, among three candidate genes, the *sll0933* gene would be involved in the functional maintenance of the PSI complex.

Introduction

All of the ecosystem of the earth surface is maintained by the light energy through the action of photosynthesis. The conversion from the sun light energy to chemical energy is carried out in photosynthetic electron transport system in plants, algae and cyanobacteria. Oxygenic photosynthetic electron transport system consists of photosystem II, plastoquinone pool, cytochrome b_6/f complex, plastocyanin or cytochrome c_6 and photosystem I.

The photosystem I (PSI) complex is a pigment-protein complex in the thylakoid membranes of cyanobacteria and chloroplasts, and consists of 11 core subunits in cyanobacteria and 15 core subunits in chloroplasts of higher plants. The PSI complex mediates the light-induced electron transfer from reduced plastocyanin or cytochrome c_6 to oxidized ferredoxin. In chloroplasts of higher plants, this complex consists of at least 5 chloroplast-encoded subunits and 8 nuclear-encoded subunits as well as more than one hundred molecules of photosynthetic pigments, phylloquinone molecules and three distinctive Fe-S clusters.

The biosynthesis of the PSI complex depends on the coordinated expression of nuclear and chloroplast genes, the targeting of the subunits to their proper location within the chloroplasts, the association of the various pigments and redox cofactors, and the proper assembly of the subunits to form an active complex. It is reasonable to assume that a lot of factors without structural function are essential to regulate the biosynthesis of PSI.

For instance, it has been reported that Ycf3 and Ycf4 are not required for the synthesis of PSI subunits, but are most likely involved in the assembly and/or stability of the PSI complex (Boudreau et al 1997). Ycf3 was shown to interact with PsaA and PsaD, whereas Ycf4 is associated with a high molecular mass complex containing several PSI subunits (Naver et al. 2001, Rochaix, J. D. et al. 2004). The *ycf4* mutant from *Synechocystis* sp. PCC6803 still has functional PSI complex but in lower amounts (Wilde, A et al. 1995). Inactivation of the cyanobacterial *ycf4* and *ycf37* decreases the PSI/PSII ratio without any influence on photoautotrophic growth (Wilde, A et al. 1995, 2001).

Ycf3, Ycf4 and Ycf37 are conserved chloroplast open reading frames, and present in higher plants, algae and cyanobacteria. Among genes of the cyanobacterial origin, however, a large number of genes have transferred to the nucleus of the host cell in evolutionary process. Only about 10% of the genes in cyanobacteria have orthologs in chloroplast genome. It is also well known that a number of nuclear genes encode chloroplast proteins that are related to photosynthesis or chloroplast biogenesis (Sato 2001, Bowman et al. 2007). Such common genes in cyanobacteria and chloroplasts show a high degree of evolutionary conservation and have potential functional importance.

In order to identify nuclear-encoded chloroplast proteins of endosymbiont origin (abbreviated as CPRENDOs: Sato et al. 2005), a comparative analysis of the genomes was carried out focusing on the relationship of orthologous genes (Sato et al. 2005). This analysis consists

of bioinformatic, large-scale estimation and experimental verification of putative CPRENDOs. As a result of investigation of growth and pulse amplitude modulation (PAM) fluorescence measurements, among the 40 disruptants of *Synechocystis*, 22 showed phenotypes related to photosynthesis (Ishikawa et al. 2009). The some of CPRENDO genes have been already annotated as ycf because they are encoded in the chloroplast genome in arbitrary photosynthetic organism (Sato et al. 2006). However, the investigation of CPRENDO would be more suited to search for novel and important genes that have been conserved in wide range of organisms than that of ycf, considering that many genes of endosymbiont origin have transferred to the nucleus of the host cell and could not be found in chloroplast genome.

In this study, from among 22 mutants of CPRENDOs of which phenotypes related to photosynthesis, I selected *sll0933, slr0815 and slr0589* mutants which were expected to have abnormality in the downstream of the photosynthetic electron transport. I aimed to identify genes which are involved in the functional maintenance of the PSI complex by investigating *sll0933, slr0815 and slr0589* mutants.

Materials and Methods

Strains and Growth Conditions

Synechocystis sp. PCC6803 wild-type (WT) and mutant strains were grown in BG-11 medium (Rippka et al., 1979) with 10 mM TES. Solid medium was supplemented with 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate. Cells in liquid culture were grown at 30°C in 50 mL glass tubes and bubbled with air under continuous illumination provided by fluorescent lumps. Photon flux density at 20μmol $m^{-2}s^{-1}$ and 200 μmol $m^{-2}s^{-1}$ were regarded as low light (LL) and high light (HL), respectively. Mutants were usually maintained with kanamycin at the final concentrations of 20μg ml^{-1}.

PAM analysis of Chlorophyll Fluorescence

Pulse-amplitude modulation (PAM) fluorescence analysis was carried out with PAM fluorometer (PAM 101/102/103, Heinz Waltz) as described in (Sonoike et al. 2001). Exponentially growing cells (OD730 ~0.5) were dark adopted for 5 min and then the measuring light was turned on to obtain the minimal fluorescent level (Fo). The fluorescence level with fully reduced Q_A (Fm') was obtained by applying multiple turnover flashes (XMT-103, Heinz, Effeltrich, Germany). The stable level of fluorescence (Fs) was determined during the exposure of cells to actinic light (KL 1500, Schott, Wiesbaden, Germany) with defined photon flux density. The far-red light that was supplied from a photodiode (FR102, Heinz Waltz, Effeltrich, Germany) was applied just after turning off the actinic light to determine

Fo'. The maximum fluorescence (Fm) was obtained by adding 10 μM DCMU to the cells under the actinic light. Fv and Fv' were defined as Fm-Fo and Fm'-Fo', respectively. Photochemical quenching (qP), non-photochemical quenching (qN or NPQ) and effective quantum yield of electron transport through PSII (ΦII) were calculated as (Fm'-Fs)/(Fm'-Fo'), 1-[(Fm'-Fo')/ (Fm-Fo)], (Fm-Fm')/Fm' and (Fm'-Fs)/Fm', respectively.

Monitoring of Chlorophyll Fluorescence Kinetics

A 10 μl aliquot of cell culture, of which the absorbance at 730 nm (A_{730}) was adjusted to 0.5 by BG-11 liquid medium, was dropped on BG-11 agar plates. After incubation at 30°C under low light for 72 h or high light for 48 h, cells on agar plates were dark adapted for 15 min before the following measurements. The plates were set in a fluorescence imaging system (FluorCam 700MF, Photon System Instruments) and actinic light (200 μmol photons $m^{-2}s^{-1}$) was applied for 45 s to monitor chlorophyll fluorescence. The fluorescence intensity was normalized with the initial value at the start of actinic light.

Isolation of Thylakoid Membranes

Cells of *Synechocystis* sp. PCC 6803 were harvested and suspended in 0.4 M sucrose, 10 mM NaCl, 1mM $CaCl_2$, 0.2 mM phenylmethylsulfonyl fluorid, 5mM benzamidine, and 50mM MOPS, pH 7.0 (MOPS buffer; Sun et al., 1998). Cells were broken by a vortex mixer with grass beads for 7 times of 30 s operations with 2 min intervals on ice. Unbroken cells and

debris were removed by centrifugation at 4,700 xg for 10 min. Thylakoid membranes were pelleted by centrifugation at 140,000 xg for 45min and re-suspended in the MOPS buffer on ice.

Quantitative Determination of P700 in Thylakoid Membranes

Thylakoid membranes (6 ng chlorophyll) were solubilized with 1% β-dodecylmaltoside. The solubilized thylakoid membranes were suspended in 50 mM Tris/HCl buffer (pH 7.5), 0.5 M sodium ascorbate, 20 mM TMPD, 2 mM methyl viologen and 1 mM DCMU on ice. Concentration of P700 was determined by measuring light-induced absorbance difference at 700nm using a dual-beam spectrophotometer (Model 356, Hitachi).

Chlorophyll Fluorescence Emission Spectra at 77 K

Low temperature chlorophyll fluorescence emission spectra were recorded using a custom-made apparatus at 77 K (Sonoike and Terashima, 1994). Cells were collected and adjusted to 5 μg chlorophyll/mL in BG-11 medium. Pigments were excited with blue light passing through a filter (Corning CS 4–96). Before the measurement, cells were dark adapted for more than 10 min at room temperature to eliminate the possible effects of state transition. Chlorophyll *a* concentrations were determined after extraction with 100% methanol (Grimme and Boardman, 1972).

Results

Growth under HL condition

High light has been reported to decrease the cellular content of PSI, giving rise to an increase in the content of PSII relative to PSI (Kawamura et al. 1979). To investigate how HL affects the growth of each mutant, I observed growth of the *sll0933, slr0815* and *slr0589* mutants as well as WT under HL. When the mutant cells on plates were grown under light at 200 μmol $m^{-2}s^{-1}$, growth of the *slr0815* mutant is slower than others (Fig. 1). In the liquid culture, the growth of the *slr0815* mutant was also remarkably slow (data not shown). This result suggested that *slr0815* is important for the growth of *Synechocystis* sp. PCC6803 under HL condition. On the other hand, the growth of all mutants under low light condition was similar to WT on both solid and liquid medium.

PAM analysis to confirm the reproducibility of the previous report

I selected *sll0933, slr0815, slr0589* mutants because they were expected to have abnormality in the downstream of the photosynthetic electron transport as a result of preliminary PAM analysis (Ishikawa et al. 2009). In order to check the previous result, I performed PAM fluorescence analysis on each mutant (Table 1). Photosynthetic parameters are shown as normalized values compared with WT. After 24 h growth under 20 μmol photons $m^{-2}s^{-1}$ (LL), all mutant showed the decrease in qP values. The extent of decrease in qP is larger than that in Fv/Fm. Since decrease in qP

represents the defect in the downstream of plastoquinone pool while that in Fv/Fm represents the defect in PSII, these three mutants of *sll0933*, *slr0815*, *slr0589* are expected to have defect in the downstream of the photosynthetic electron transport. In addition, I also performed PAM fluorescence analysis with the cells grown under 200 μmol photons $m^{-2}s^{-1}$ (HL). In HL acclimated cells, *sll0933* and *slr0815* mutants showed that more decreased qP than Fv/Fm, particularly, qP of *slr0815* mutants was remarkably decreased compared to LL acclimated cells. The result of *slr0589* mutant showed increased Fv/Fm despite decreased qP than WT.

Analysis of chlorophyll fluorescence kinetics by FluorCam

Analysis of chlorophyll fluorescence kinetics by FluorCam is suitable to examine the phenotype of mutants. I first cultivated the cyanobacteria in patches with a diameter of about 1 cm on plates under 20 μmol photons $m^{-2}s^{-1}$(LL) and 200 μmol photons $m^{-2}s^{-1}$ (HL) conditions, and then the plates were dark adopted for 15 min and subjected to the measurements (Fig. 2). The change in the fluorescence intensity of *sll0933* mutant was apparently smaller than WT and other mutants under both LL and HL conditions. The fluorescence kinetics of *slr0589* mutant showed faster rise than that of WT under LL, and it was similar to WT under HL. The fluorescence kinetics of *slr0815* mutant which showed remarkably slow growth under HL had a lower first peak (at around 0.5 s after the onset of the illumination) than WT, and the final level of fluorescence (at around 0.5 s after the onset of the illumination) was descending under both LL and

HL conditions. In addition, I measured the fluorescence kinetics of *slr0815* mutant under 110 and 150 μmol photons $m^{-2}s^{-1}$ conditions. This result showed a lower second peak (at around 5 s after the onset of the illumination) under 150 μmol photons $m^{-2}s^{-1}$ condition than 110 μmol photons $m^{-2}s^{-1}$ condition. Thus, the fluorescence kinetics of *slr0815* mutant was similar to WT under middle light condition but not under LL and HL condition. This indicates that *slr0589* mutant has similar phenotype to WT only under middle light condition. Such a phenotype has not been reported in the past.

Chlorophyll Fluorescence Emission Spectra determined at 77 K

At room temperature, chlorophyll fluorescence of PSI is very weak and not able to distinguish from that of PSII. At 77 K, however, the fluorescence of PSI shows peak at 715-735 nm while that of PSII shows peak at 685 nm and 695 nm. To investigate whether PSI amounts of mutants differ from that of WT, I determined the relative ratio of PSI/ PSII by monitoring 77 K emission spectra of chlorophyll fluorescence of cells cultured under LL for 48 h or HL for 24 h (Fig. 3, Table 2). In the *sll0933* mutant grown under low light condition, the relative intensity of PSI to PSII fluorescence is similar with that in WT. In the *slr0589* and *slr0815* mutants, however, the relative intensity was greater than WT. In high light-acclimated cells, the relative intensity of PSI fluorescence of all the mutants was greater than WT. In the *sll0933* mutant under HL growth condition, the ratio of PSI/PSII was not decreased so much compared to

WT. In the *slr0589* mutant under HL growth condition, the ratio of PSI/PSII was decreased more than WT. These results indicate that relative PSI amounts of *sll0933* mutant is greater than WT only under HL growth condition, and that of *slr0815* mutant is greater than WT under both LL and HL growth conditions, and that of *slr0589* mutant is greater than WT only under LL growth condition.

The Chlorophyll a Content per Cell

Though the chlorophyll *a* is contained in both PSI and PSII, the most of chlorophyll *a* is associated with PSI because the chlorophyll *a* is main antenna pigment of PSI while phycobilin in phycobilisome mainly works for antenna of PSII. Therefore I examined the chlorophyll *a* content per cell to investigate the amount of PSI in each mutant under LL and HL growth conditions (Fig. 4). To estimate the chlorophyll content per cell, I determined the ratio of absorption at 678 nm (A_{678}) to optical density at 730 nm (OD_{730}) of absorption spectrum. In low light-acclimated cells, A_{678}/OD_{730} of all the mutants are slightly smaller than WT. In high light-acclimated cells, A_{678}/OD_{730} is twice or more higher in the *slr0815* mutant compared with the WT. A_{678}/OD_{730} of *sll0933* and *slr0589* mutants also indicated slightly larger values than that of WT. This result suggests that under HL growth condition, PSI per cell of *slr0815* mutant does not decrease so much compared to WT, and PSI per cell of *sll0933* and *slr0589* mutant slightly greater than WT.

Quantitative determination of P-700 in thylakoid membrane

P-700 is reaction center of photosystem I and consists of chlorophyll *a* and its stereoisomer chlorophyll *a*'. The determination of P-700 gives the precise quantification of PSI content. I first isolated the thylakoid membrane from the cells of mutants that grown under LL condition. The *sll0933* mutant indicated the lower content of P-700 and greater antenna size than WT under LL growth condition, and the higher content and smaller antenna size than WT under HL growth condition (Table 3). The *slr0815* mutant indicated the much lower content of P-700 and greater antenna size than WT under LL growth condition, and the slightly higher content and smaller antenna size than WT under HL growth condition.

Furthermore, result under HL growth condition was similar to result under LL. The *slr0589* mutant indicated the lower content of P-700 and higher antenna size than WT under LL growth condition like the *sll0933* mutant, and the lower content and higher antenna size than WT under HL condition. These results indicated that all mutants, particularly *slr0815* mutant has lower P-700 than WT under LL condition, and P-700 of *slr0815* mutant does not decrease so much under HL condition.

Discussion

To date, several genes, which are necessary for the functional maintenance of PSI but not the components of PSI, have been identified. It has been reported that Ycf3 and Ycf4 are most likely involved in the assembly and/or stability of the PSI complex, and function as chaperons during PSI complex formation (Boudreau et al 1997). It has been also reported that Ycf37 has a functional role in PSI assembly and/or stability, and inactivation of *ycf37* leads to a lower PSI/PSII ratio in *Synechosystis* cells (Wilde, A et al. 2001). However, there are many nuclear-encoded and unidentified proteins. I assume that, among them, there are many factors involved in the biogenesis or maintenance of PSI. In this study, I explored genes which are involved in the functional maintenance of the PSI complex by investigating the mutants of three genes, *sll0933, slr0815 and slr0589*.

To study the function of *sll0933, slr0815 and slr0589,* first I examined if these genes have abnormality in the downstream of the photosynthetic electron transport by PAM fluorescence analysis. As shown in the Results section, all the mutants were expected to have defect in the downstream of the photosynthetic electron transport, supporting the previous report (Ishikawa et al. 2009). However, it does not necessary mean that the defect is in PSI. Therefore, I examined the function and quantity of PSI by several different approaches.

Chlorophyll fluorescence emission spectra determined at 77 K gives

the information on PSI/PSII ratio. In LL acclimated cells, PSI/PSII ratio of only *sll0933* mutant was decreased compared to WT. However, this result does not necessarily indicate decrease of PSI because PSII might have increased. In cyanobacteria, most of the chlorophyll (usually about 90% of total chlorophyll) is associated with PSI. Phycobilisome, instead of chlorophyll, usually serves for main antenna of PSII. Thus, chlorophyll content per cell could be used for the estimation of PSI content in cells. All the mutants have lower value of chlorophyll/cell in LL acclimated cells. As another approach, I quantified P-700, the reaction center chlorophyll of PSI. P700 content gives the precise quantification of PSI reaction center. The *sll0933* mutant indicated the lower content of P-700 and greater antenna size than WT under LL growth condition. Thus, the *sll0933* mutant grown under LL showed low PSI/PSII, low Chl/Cell, and low P-700 content. This result apparently indicated that PSI content of the *sll0933* mutant decreased when compared with WT. The *ycf4* and *ycf37* mutants were also reported as the 'low PSI' mutants. Considering that the chlorophyll fluorescence kinetics of the *sll0933* mutant was similar to that of *ycf4*, I assume that Sll0933 might be involved in assembly and/or stability of PSI. However, there is no direct evidence to prove the hypothesis at present, and this is the problem that should be solved in future.

The content of P-700 of the *slr0815* mutant was much decreased under LL condition compared to WT. However, the content was not decreased under HL condition. Because the *slr0815* mutant indicated higher PSI/PSII and Chl/Cell under HL condition, it was suggested that the

slr0815 mutant has abnormality in the antenna. In the WT, PSI decreased under HL condition in order to avoid excessive energy. Considering the increase in chlorophyll antenna of the *slr0815* mutant and slow growth under HL condition, it is possible that Slr0815 is involved in the regulation of photosystem stoichiometry under high light condition.

The slr0589 mutant grown under LL indicated the lower content of P-700 and Chl/Cell than that of WT. The mutant, however, showed higher PSI/PSII ratio compared with WT in the measurements of chlorophyll fluorescence spectra at 77K. Some modification at the level of chlorophyll molecules might influence the chlorophyll fluorescence spectra. Thus, more detailed analysis is required to reveal the function of the *slr0589* mutant.

In conclusion, among three genes of CPRENDOs, I could indicate that only *sll0933* is involved in the functional maintenance of the Photosystem I.

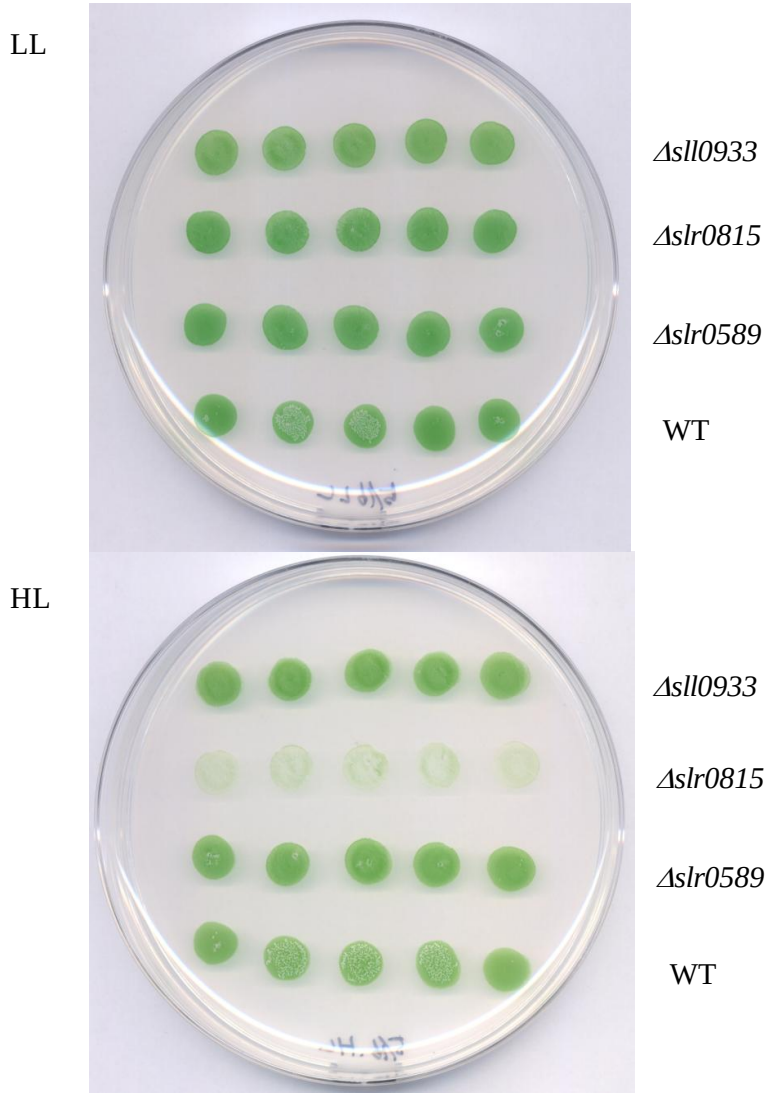


Fig. 1 Growth of mutants and WT under LL and HL condition
A 10 ml aliquot of liquid cultured cells (OD730=0.5) grown under LL conditions were spotted on BG-11 plates by five spots. The cells were grown under 20 μmol photons $m^{-2}s^{-1}$(LL) for 72h and 200 μmol photons $m^{-2}s^{-1}$ (HL) for 48h.

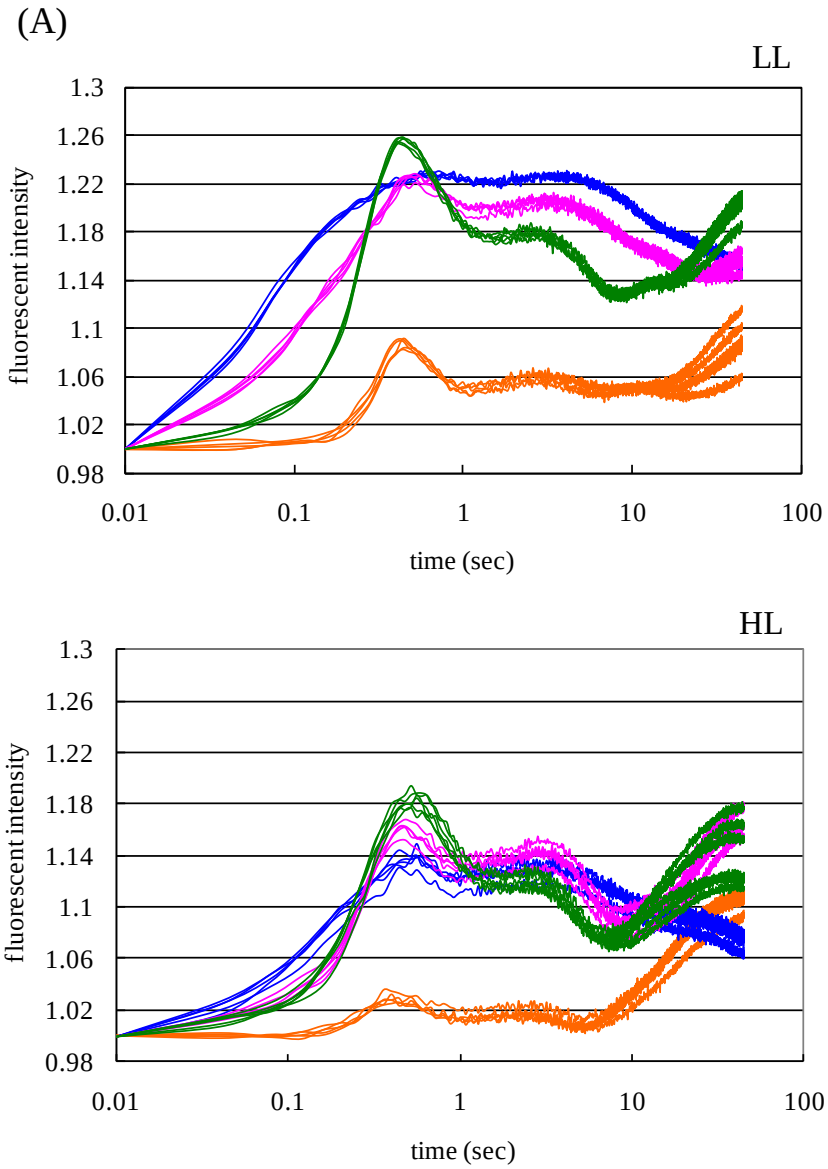
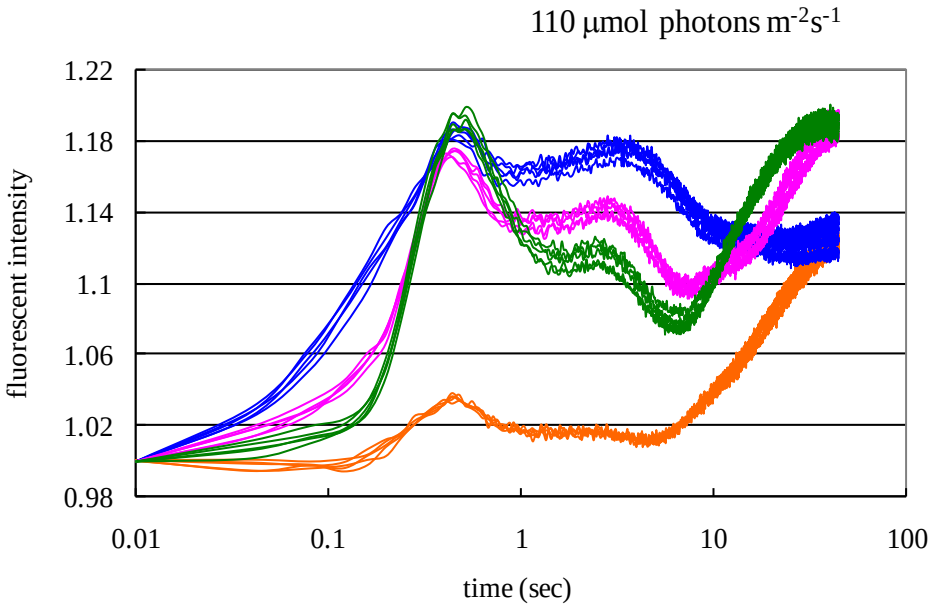


Fig. 2 Chlorophyll fluorescence kinetics of mutants and WT
The cells of *sll0933* (orange), *slr0815* (blue) and *slr0589* (pink) mutants and wild type (green) were grown under 20 μmol photons $m^{-2}s^{-1}$ (LL) for 72h and 200 μmol photons $m^{-2}s^{-1}$ (HL) for 48h (A), under 110 μmol photons m-2s-1 and 150μmol photons m-2s-1 for 48h (B). The cells on BG-11 plates were illuminated with at 200 μmol photons $m^{-2}s^{-1}$ for 45s to monitor fluorescence kinetics after 15 min dark adaptation. The fluorescence intensity was normalized with the initial value at the start of actinic light.

(B)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1.02
0.98
fluorescent intensity
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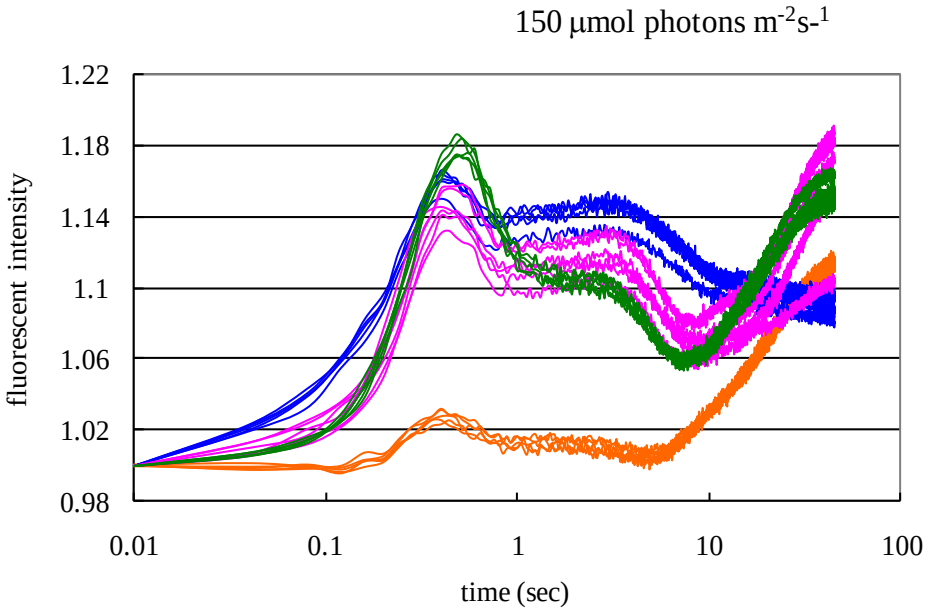
0.1
1
10
100
time (sec)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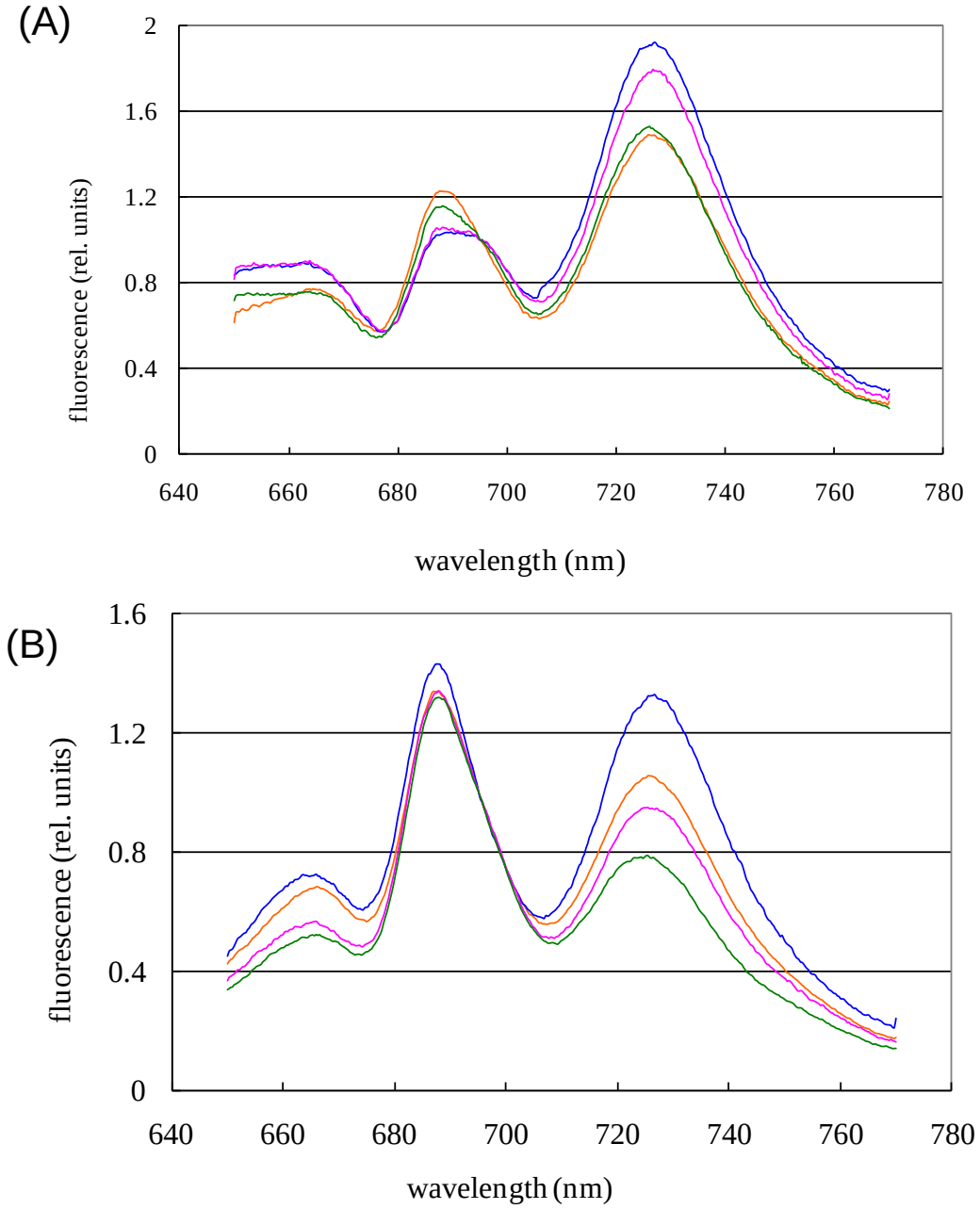

Fig. 3 77K fluorescence emission spectra
The 77K fluorescence emission spectra of *sll0933* (orange), *slr0815* (blue) and *slr0589* (pink) mutants and wild type (green) grown under LL (A) and HL (B) condition. The chlorophyll concentrations were adjusted to 5 mg/ml.
The values were normalized to emission at 695nm.

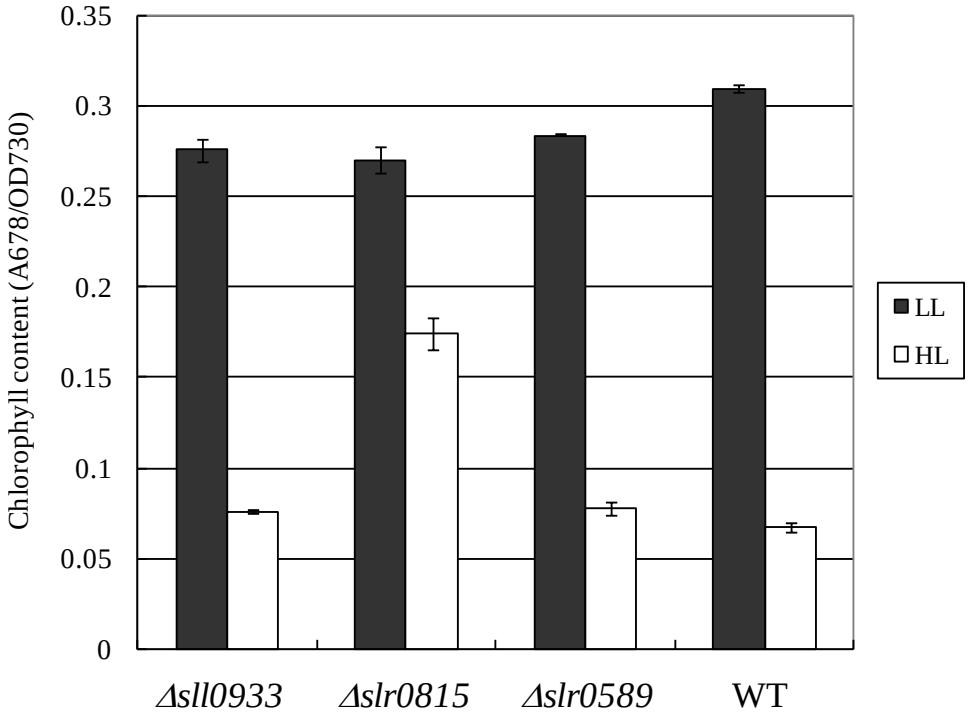

Fig 4. Chlorophyll content per cell

Chlorophyll content per cell was estimated from absorption spectrum at 678 nm that is the maxima of chlorophyll a absorption and OD730 of grown cells for 24 h under 20 μmol photons $m^{-2}s^{-1}$(LL) and 200 μmol photons $m^{-2}s^{-1}$ (HL) conditions. Each bar represents the SD of results of experiments on three times.

(A) LL acclimated cells PAM

Gene	qP	qN	ΦII	NPQ	Fv/Fm	Fv'/Fm'
WT	0.62	0.17	0.27	0.09	0.49	0.44
	100%	100%	100%	100%	100%	100%
Δsll0933	48%	229%	26%	122%	73%	55%
Δslr0815	92%	135%	93%	167%	98%	98%
Δslr0589	94%	165%	85%	178%	96%	98%

(B) HL acclimated cells PAM

Gene	qP	qN	ΦII	NPQ	Fv/Fm	Fv'/Fm'
WT	0.74	0.74	0.14	0.50	0.50	0.20
	100%	100%	100%	100%	100%	100%
Δsll0933	42%	49%	44%	36%	55%	107%
Δslr0815	26%	82%	35%	82%	96%	136%
Δslr0589	77%	85%	148%	131%	121%	190%

Table 1 PAM parameters of mutants and WT

The cells were grown under 20 μmol photons $m^{-2}s^{-1}$(A) and 200 μmol photons $m^{-2}s^{-1}$(B) for 24h and exponentially growing Cells (OD730 ~0.5) were dark adopted for 5 min. PAM parameters are indicated as the absolute values of wild type and percentage normalized to values of wild type.

	Δsll0933	*Δslr0815*	*Δslr0589*	WT
LL	1.271	1.852	1.697	1.321
HL	0.784	0.929	0.706	0.598

Table 2 PSI/PSII ratio of fluorescence emission spectra at 77 K

PSI/PSII ratio was calculated from maximum values at PSI and PSII peaks in the 77 K emission spectra of chlorophyll fluorescence.

(A)

	Δsll0933	*Δslr0815*	*Δslr0589*	WT
P700 (mM)	4.13×10^{-5}	3.25×10^{-5}	4.05×10^{-5}	4.60×10^{-5}
Chl/P700	145	184	148	130

(B)

	Δsll0933	*Δslr0815*	*Δslr0589*	WT
P700 (mM)	3.81×10^{-5}	3.33×10^{-5}	3.10×10^{-5}	3.45×10^{-5}
Chl/P700	158	180	194	174

Table 3 Quantitative determination of P-700
Samples were measured after isolation of thylakoid membranes from grown cells under 20 μmol photons $m^{-2}s^{-1}$(A) and 200 μmol photons $m^{-2}s^{-1}$ (B) and the chlorophyll concentration was adjusted to 6 nM. P-700 was calculated from difference of measured absorbance at 700nm between reduced and oxidized P-700.The antenna size (Chl/P-700) of PSI was calculated from the value of P-700 and chlorophyll concentration.

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