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利用基因剔除技術研究藍綠菌*Synechococcus* sp. PCC 7335中可吸收
遠紅光的光系統一

Mutagenesis study of the Photosystem I that absorbs far-red light in a
cyanobacterium *Synechococcus* sp. PCC 7335

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7335

本論文係陳楷文君 (R09B21026) 在國立臺灣大學生命
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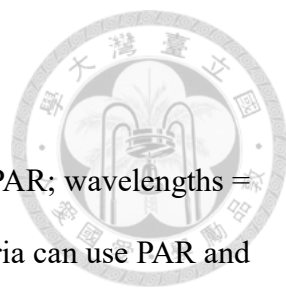


摘要

大部分的光合生物可以利用波長 400-700 奈米的可見光能源。然而，有一些品種的藍綠菌除了可以利用 400-700 nm 波長的能源外，也可以利用遠紅光（波長 700-800 奈米）。當生活在可見光較少且遠紅光較多的環境時，它們可以行遠紅光轉換作用來適應環境。經由遠紅光轉換，這些藍綠菌會產生葉綠素 *d*、*f*，以及表現遠紅光基因簇中的 20 個基因來重組光系統一、二以及藻膽蛋白體。近期冷凍電子顯微鏡解出的藍綠菌遠紅光下的光系統一結構，揭露了幾個可能的葉綠素 *f* 結合位點。然而，葉綠素 *f* 以及葉綠素 *a* 結構上非常相似，因此以目前的技術要能夠直接區分出兩者具有非常大的挑戰性，在一些解析度比較侷限的區域上更是不容易確定。並且，每個葉綠素 *f* 在光系統一中所扮演的角色也不清楚。為了彌補結構研究上的不足，我們選定了在遠紅光光系統一中坐落在可能葉綠素 *f* 結合位點附近的四個基因(*psaF2*, *I2*, *J2*, and *L2*)來進行基因剔除。結果顯示 *psaL2* 突變株不但在遠紅光下長得比較慢，而且同時他們的葉綠素 *f*:*a* 比例也降低了。此外我們也發現缺少 *PsaF2* 和 *PsaJ2* 並不會影響到光系統一的三聚體結構，但卻會改變光譜特性。再者，結合先前的結構研究以及以高效液相層析測量得出的結果—在遠紅光下突變株 *psaJ2* 光系統 I 中的葉綠素 *f* 含量比率和野生株以及突變株 *psaF2* 的光系統 I 中的含量來的低可以推斷出，在蛋白為單元體 *PsaJ2* 附近有一個葉綠素 *f* 的結合位。

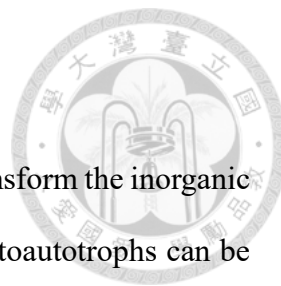
關鍵字：遠紅光轉換，基因剔除，螢光光譜學，吸收光譜學

Abstract



Most oxygenic phototrophs use photosynthetically active radiation (PAR; wavelengths = 400-700 nm) for photosynthesis. However, some special cyanobacteria can use PAR and far-red light (FRL, wavelengths = 700-800 nm). When these cyanobacteria grow in FRL-enriched environments, they perform far-red light photoacclimation (FaRLiP) to harvest FRL. While performing FaRLiP, they synthesize chlorophyll *d* (Chl *d*) and chlorophyll *f* (Chl *f*) and express 20 genes in the FaRLiP gene clusters to remodel their photosystem I (PSI), photosystem II, and also phycobilisome. Recent PSI structural cryogenic electron microscopy (cryo-EM) studies identified several possible Chl *f* binding sites in the FRL-remodeled PSI. However, the structure of Chl *f* and Chl *a* are too similar, and in some regions with lower resolution, they are hard to differentiate under the resolution of cryo-EM. In addition, the function of each Chl *f* molecule in PSI is still unknown. To complement the deficiency of cryo-EM, we individually used conjugation and homologous recombination to knockout the four PSI subunits (PsaF2, I2, J2, and L2) in the FaRLiP gene cluster in *Synechococcus* sp. PCC 7335. These four subunits are near the proposed binding sites in the cryo-EM structure. We found that the *psaI2*⁻ and *psaL2*⁻ mutant grows slower than the wild type under FRL and decreases the Chl *f*: *a* ratio. Also, the PSI purification result shows that the lack of PsaF2 or PsaJ2 does not affect trimerization but changes the spectral properties of the complex. Moreover, combined with previous structural studies and the HPLC analysis that the Chl *f* molecules ratio is decreased in the purified FRL PSI of *psaJ2*⁻, there is a Chl *f* binding site near PsaJ2.

Keywords: far-red light photoacclimation (FaRLiP), gene knockout, fluorescence spectroscopy, absorption spectroscopy



1. Introduction

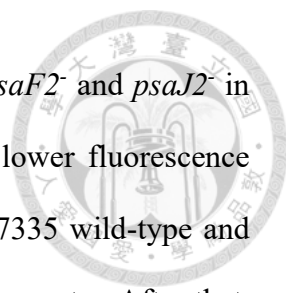
On earth, all photoautotrophs can acquire energy from the sun and transform the inorganic compounds into organic compounds as food sources to survive. Photoautotrophs can be further classified into monoplast and multicellular organisms. Among all the photoautotrophs, cyanobacteria are the most primitive and have simple physiological regulation mechanisms, becoming one of the best model organisms to study physiological mechanisms and gene regulations (Whitton, 2012). When cyanobacteria grow in the wild, they will encounter many survival competitions to strive for limited sources such as light, carbon, and organic compounds. In the competition process, superior cyanobacteria can occupy the upper niche close to the top of the surroundings to acquire more light sources. Instead, some cyanobacteria that are not superior to others can only live in deeper places that have a limited visible light source. (Kardinaal et al., 2007) However, under this circumstance that the far-red light can reach a deeper region (Gan & Bryant, 2015). And that is this situation that caused the cyanobacteria to evolve the capability to use far-red light, which is later named far-red light photoacclimation (Gan et al., 2014; Wolf & Blankenship, 2019), to help them acclimate to an environment that has more far-red light (FRL) and less photosynthetically active radiation (PAR; 400 – 700 nm).

In this study, a cyanobacterial strain, *Synechococcus* sp. PCC 7335, which can perform far-red light photoacclimation (FaRLiP), was chosen to investigate the FaRLiP mechanism. (Ho et al., 2017). When the cyanobacteria which can conduct FaRLiP grows under an environment having more FRL and PAR, the P_{fr} form of RfpA would receive the light and then activate the downstream FaRLiP gene cluster and express 20 genes to remodel the photosystem II (PSII), photosystem I (PSI), and phycobilisome. Also, when the cyanobacteria which can perform FaRLiP acclimated to far-red light, they can produce chlorophyll *d* (Chl *d*) and chlorophyll *f* (Chl *f*) (Ho et al., 2016). And Chl *d* and Chl *f*

molecules can bind to the remodeled PSI and PSII and help the cyanobacteria acquire far-red light energy. With the help of Chl *d* and Chl *f* molecules binding in the proper position on PSII and PSI, cyanobacteria can use the FRL energy efficiency and make them able to survive (Chen et al., 2002; C. Gisriel et al., 2020).

Based on previous studies, the enzyme capable of producing Chl *f* molecules was found (Ho et al., 2016), but the binding sites of the Chl *f* molecules were unclear. Also, because each photon of FRL energy is less than the photon that comes from PAR, identifying the locations of Chl *f* molecules is a vital issue in knowing the uphill energy transfer steps and the mechanism of FaRLiP. In the previous studies, the Brudvig group from Yale University used cryogenic electron microscopy (cryo-EM) to identify the PSI structure fully acclimated to FRL (Christopher Gisriel et al., 2020; Christopher J. Gisriel, David A. Flesher, et al., 2022; Gisriel et al., 2021). Based on their cryo-EM data, the authors proposed several possible Chl *f* molecules binding sites in their protein complexes' results. However, the slight functional differences between Chl *a* and Chl *f* molecules, which have a methyl group on the C2 position and a formyl group, make them hard to differentiate under cryo-EM (**fig. 1B, 1C**). Also, when the cyanobacteria perform FaRLiP, the FaRLiP gene cluster would be expressed, among them, six PSI genes (*psaA2*, *psaB2*, *psaF2*, *psaI2*, *psaJ2*, and *psaL2*) would remodel PSI to help the cyanobacteria live under this condition (**fig. 2**).

In this study, some subunits (PsaF2, PsaI2, PsaJ2, PsaL2) near the possible Chl *f* molecules binding sites were chosen to do the mutagenesis to identify the potential critical areas of Chl *f* molecules (**fig. 3**). The knockout mutants were further examined by colony PCR and sent for sequencing. After that, they were cultured in a liquid ASN-III medium for growth curve, absorbance spectrum, and fluorescence spectrum measurements. In the growth curve measurement, *psaI2*⁻ and *psaL2*⁻ show that they have a longer doubling time



than *Synechococcus* sp. PCC7335 (Syn7335) wild-type (WT) and *psaF2⁻* and *psaJ2⁻* in far-red light. And the two mutants (*psaI2⁻* and *psaL2⁻*) also have lower fluorescence emission and FRL absorbance ability than *Synechococcus* sp. PCC7335 wild-type and *psaF2⁻* and *psaJ2⁻* in far-red light under fluorescence and absorbance spectra. After that, the four mutants (*psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, and *psaL2⁻*) and WT were cultured in large volumes to further PSI isolation. PSII and PSI were purified and separated with a 5-20% sucrose gradient. The isolation results show that PSI trimer only can be found from FRL Syn7335-WT, *psaF2⁻*, *psaJ2⁻* but not on *psaI2⁻* and *psaL2⁻*. The further PSI trimers purified from the FRL Syn7335-WT, *psaF2⁻*, and *psaJ2⁻* were used to detect the protein complexes' absorbance and fluorescence spectra and the HPLC analysis. In the absorbance and fluorescence spectrum, *psaF2⁻* and *psaJ2⁻* have a blue shift Q_y peak compared with FRL-WT PSI. And the peak height of around 730 nm of *psaJ2⁻* seems to be higher than that of *psaF2⁻* under the room-temperature (RT) fluorescence spectrum. Besides, *psaI2⁻* and *psaL2⁻* did not have a band containing a trimeric structure of PSI. This result is similar to a previous study that knocked out white light type *psaI2* and *psaL2* (Schluchter et al., 1996). In the HPLC analysis, the Chl *f* content seems to be the same between the cells' FRL-WT, *psaF2⁻*, and *psaJ2⁻*. However, the Chl *f* ratio is lower in the purified PSI of *psaJ2⁻*. In this study, we found that knockout of *psaI2* and *psaL2* would affect the growth of Syn7335 under FRL but not under WL. Second, knockout of *psaI2* or *psaL2* would affect the PSI structure of Syn7335 under FRL and cause trimeric PSI cannot be generated. Third, combined with the previous structural study and the HPLC analysis, there is a Chl *f* binding site around PsaJ2.



2. Material and methods

2.1 Cyanobacterial strains and growth conditions

The cyanobacteria strain *Synechococcus* sp. PCC 7335 (Syn7335) was taken from Pasteur Culture Collection Center (www.pasteur.fr/en/pcc). Syn7335 originated on the snail shell in the intertidal zone close to Puerto Penasco, Mexico (Rippka et al., 1979). The strain was cultured in ASN-III medium replenished with vitamin B₁₂ (4 mg L⁻¹) and 10 mM Tris-HCl, pH 8.0 at 30°C, supplemented with 1% (v/v) CO₂. The composition of the modified ASN-III medium is shown in **appendix table 1**. Continuous illumination of white LED light (HiPoint, Taiwan) with ~100 μmol photons m⁻² s⁻¹ and far-red LED light (HiPoint, Taiwan) with ~100 μmol photons m⁻² s⁻¹ (centered at 730 nm) were used as the light sources to culture the cyanobacteria.

2.2 Cargo plasmid building up

The plasmid pRL277 was double cut by SphI, and the digested product with 5901bp was set as the cargo plasmid backbone. BamHI and SpeI digested the pJET-km, and the *aphAII* resistant cassette was used as the insert. The other two inserts of the cargo plasmid on the target genes' upstream and downstream 3 kb of Syn7335 genomic DNA were amplified with the primer containing 29-30 bp overhang with digested pRL277 backbone and *aphAII* resistant cassette. Then the four segments were assembled by the HIFI DNA assembly (NEB, USA), and the product was used to transform into *E. coli* DH5α. All of the primers used for the construct building up are shown in **appendix table 2**.

2.3 Conjugative transformation and homologous recombination in *Synechococcus* sp. PCC 7335

The system of conjugation exerted on *Synechococcus* sp. PCC 7335 was adapted from previous studies with slight changes (Zhao et al., 2015; Zhao et al., 2005). DNA fragments (~3 kb) from the upstream and downstream flanking region of the target gene (*psaF2*, *psaI2*, *psaJ2*, *psaL2*) were amplified by polymerase chain reaction (PCR). And the *aphAII* encoding kanamycin-resistant cassette was digested with BamHI and SpeI from the plasmid of pJET-km. Also, a ~7kb fragment taken from pRL277 was digested with SphI and set as the backbone of the cargo plasmid. All four segments were linked with HIFI DNA assembly (NEB, USA) into one intact cargo plasmid. The cargo plasmids were further transformed into *Escherichia coli* HB101 competent cells containing conjugal plasmid pRL443 and helper plasmid pRL623 (Elhai et al., 1997; Elhai & Wolk, 1988). *E. coli* cells were cultured at 37°C for 16-18 h in 20 mL of Luria-Bertani (LB) medium supplemented with 100 mg ampicillin L⁻¹, 50 mg chloramphenicol L⁻¹, 100 mg spectinomycin L⁻¹, 12.5 mg tetracycline L⁻¹, and 100 mg kanamycin L⁻¹. 20 mL of *E. coli* culture and 5 mL of *Synechococcus* sp. PCC 7335 cells (OD_{750 nm} = 0.4-0.8) were centrifugated at 5510 x g for 5 min. *E. coli* cell pellets were washed with LB medium twice, ASN-III medium once, and *Synechococcus* sp. PCC 7335 cell pellets were washed with ASN-III medium three times. After washing the cell pellets, *Synechococcus* sp. PCC 7335 cells pellets were resuspended with 200 µL of ASN-III medium. Further, the solution containing Syn7335 cells was used to resuspend the *E. coli* cell pellets. Then the mixtures were left at room temperature for 4-6 h under low light and streaked on a sterile nitrocellulose filter (diameter = 47 mm; pore size = 0.45 µm, PALL, USA) overlaid on a 1.5% (w/v) ASN-III agar plate. After staying under low light for two days, the nitrocellulose membrane was transferred to a 1.5% (w/v) ASN-III agar plate

supplemented with kanamycin (100 mg L^{-1}). After colonies became observable on the plate, they were picked up and streaked on a new ASN-III plate supplemented with kanamycin (100 mg L^{-1}). Colony PCR using the primers, which amplified partly of the upstream and downstream flanking region of the target gene covered with *aphII* antibiotic-resistant cassette, was performed to confirm the genetic knockout of the conjugants.

2.4 Absorption spectroscopy and room temperature and low-temperature (77K) fluorescence spectroscopy

WT and mutant strains (*psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, *psaL2⁻*) of Syn7335 were cultured in WL and FRL until the $\text{OD}_{750 \text{ nm}}$ values reached 0.5-1.0. Aliquots of cells were collected by centrifugation with $10000 \times g$ for 2 min. An Agilent spectrometer (Cary 60, Agilent, USA) detected the $\text{OD}_{750 \text{ nm}}$ value, and the cell density was adjusted to $\text{OD}_{750 \text{ nm}} = 0.2$ with a total volume of 1 mL in a modified ASN-III medium to measure the absorption spectrum for cells with MAPADA (UV-1800, China). On the other hand, to measure the absorption spectrum of the purified proteins, the purified protein products were dissolved in MES buffer (50 mM MES, pH 6.5, 10 mM CaCl_2 , and 10 mM MgCl_2) with absorbance between 400 – 700 nm less than 1.0, and the spectrum was detected by Agilent spectrometer. The fluorescence spectra were all measured with the Hitachi F7000 fluorometer (Hitachi, Japan), but the protocols were slightly different when detecting cells and purified protein products. The machine was set at a PMT voltage of 700V with an excitation slit and emission slit of 5 nm. All the fluorescence spectra were detected with a scan speed of 240 nm min^{-1} . The cell density was also adjusted to $\text{OD}_{750 \text{ nm}} = 0.2$ with a total volume of 2 mL in a modified ASN-III medium to measure the room temperature fluorescence spectrum of the cells. Instead, the chlorophyll concentration was adjusted to $10 \mu\text{g mL}^{-1}$

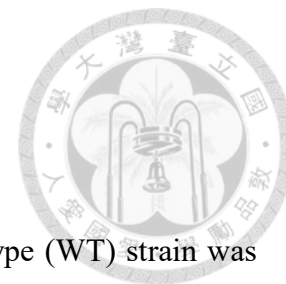
in MES buffer to measure the room temperature spectrum of purified proteins. The cells were centrifugated into pellets in microcentrifuge tubes first, and then 500 μL of modified ASN-III medium was used to resuspend the cells. 500 μL of 30 % (w/v) PEG as a cryoprotectant was added for 77K fluorescence spectroscopy. To measure purified protein 77K fluorescence spectrum, the PSI should be dissolved in 50 mM MES buffer, and the total chlorophyll concentration should reach 30 $\mu\text{g/mL}$ in 60% glycerol (v/v) solution.

2.5 Pigments extraction and HPLC analysis

Cyanobacteria cells were centrifugated with 13000 x g for 2 min to collect cell pellets. The upper layer supernatant was discarded, and then the cell pellets were washed with 10 mM Tris-HCl, pH 8, once and then centrifugated with 13000xg for 2 min to remove the supernatant. After that, the cell pellets were added with 270 μL of acetone/methanol (7:2, v/v) and vortexed for 2 min to resuspend the pellets and mix with the solution thoroughly. Later, the solution should be centrifugated to remove the cell debris, and then the supernatant was left and then filtrated with a 0.2 μm pore size of polytetrafluoroethylene membrane syringe filter (PALL, USA). The flow-through solution was added with 1/10 volume of 1M ammonium acetate (NH_4OAc) and then transferred into the vials with caps. A JASCO PU-4180 RHPLC system (JASCO, Japan), matched with an analytical Discovery C18 column (25 cm x 4.6 mm) (KANTO CHEMICAL, Japan), was used to analyze the pigments. The method of reversed-phase HPLC analysis was performed as previously described (Gan & Bryant, 2015; Gan et al., 2014; Ho et al., 2016; Zhao et al., 2015).

2.6 Isolation of trimeric PSI complexes

As previously described, PSI complexes were isolated from the cells grown in FRL (Christopher Gisriel et al., 2020; Kurashov et al., 2019; Shen et al., 2019). The cells were collected by centrifugation at 10000 x g for 10 min and then washed with 50 mM Tris, pH 8 twice. The cells were then resuspended with 13 mL of MES buffer. The cells pellets were then added into a bead beater container with 0.05 mm and 0.01 mm glass beads with 1/4 volume of the cell solution and underwent beating by a bead-beater (BioSpec Products, USA) with [2 min beating, 1 min break] x 6 cycles. After six cycles of bead beating, the solution was transferred to 50 mL falcon tubes for centrifugation (10000 xg, 5 min) to remove cell debris. And then, the supernatant was further transferred into a 13PA centrifuge tube (Hitachi, Japan) and underwent ultracentrifugation with 126100 x g (CR22N, Hitachi, Japan) at 4°C for 30 min to collect the thylakoids. After centrifugation to remove sizeable insoluble debris, the thylakoid membrane was dissolved with 3 mL of MES buffer and then homogenized with Potter-Elvehjem homogenizer. The homogenized membrane was then transferred into a 15 mL centrifuge tube and added n-dodecyl- β -D-maltoside (β -DM) until 1% of the total solution (v/v). The homogenized membrane was then continuously rotated for 1 h at 4 °C. After solubilization, the solution was further centrifuged to remove the insolubilized membrane and loaded onto a 40 PA centrifuge tube (Hitachi, Japan) containing (5 – 20% (w/v) sucrose, 0.01% β -DM (v/v)) sucrose gradient for 139000 x g, 18 h, 4°C ultracentrifugation (CR22N, Hitachi, Japan). Green-colored, chlorophyll-containing fractions representing trimeric FRL-PSI were collected and dialyzed against MES buffer. The dialyzed membrane was concentrated with a 30 kDa concentrator (Sartorius, Germany), and further dimethyl sulfoxide (0.5% (v/v)) and glycerol (10% v/v) were added to the purified complexes for crypto conservation. The samples were stored at -80°C until further spectroscopy and pigment analyses.



3. Results

3.1 Conjugants built up

The cyanobacteria *Synechococcus* sp. PCC 7335 (Syn7335) wild-type (WT) strain was set as the PCR template to amplify the target genes' upstream and downstream 3 kb segments. And these two flanking segments were linked with kanamycin-resistant cassette *aphAII* and pRL277 backbone to form thorough cargo plasmids, as shown in **fig. 4A**. Later, the cargo plasmids targeting *psaF2*, *psaI2*, *psaJ2*, and *psaL2* were utilized to do homologous recombination in Syn7335 and then replace the target genes with kanamycin-resistant cassette *aphAII* as shown in **fig. 4A**. Further, the conjugants were specified with primers amplified partly of the flanking region of target genes covered with *aphAII* antibiotic resistant cassette as shown in **fig. 4B**. And in **fig. 4C**, the knockout mutants of *psaF2*, *psaI2*, *psaJ2*, and *psaL2* were generated and verified.

3.2 The comparisons of growth rates

After the specification of colony PCR, each of the three correct conjugants of the mutants was picked up as three independent clones representing each mutant for growth curve and doubling time measurements. The growth curves shown in **fig. 5** indicate the growth curves of Syn7335 WT and the mutants *psaF2*⁻, *psaI2*⁻, *psaJ2*⁻, and *psaL2*⁻ under visible light (WL) and far-red light (FRL), respectively. As the curves show, all the groups seem to have similar growth rates under WL, but *psaI2*⁻ and *psaL2*⁻ had a slower growth rate than WT, *psaF2*⁻, and *psaJ2*⁻ under the FRL condition. **Fig. 6** shows that the doubling time of *psaI2*⁻ and *psaL2*⁻ have a longer time to double their cell number when compared with other mutants and WT. Also, the doubling time of the Syn7335 WT shows a significant difference in statistics (p-value < 0.05, Kruskal Wallis test) compared with *psaI2*⁻ and *psaL2*⁻ under FRL. This result indicated that losing PsaI2 or PsaL2 would be

critical to the growth of Syn7335 while growing in FRL.



3.3 Absorption spectra of Syn7335 WT, *psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, *psaL2⁻* cells grown under FRL

In the absorption spectra (**fig. 7A**) of the FRL cells, *psaF2⁻* and *psaJ2⁻* have higher FRL absorbance peaks around 710 nm compared with FRL-WT, *psaI2⁻* and *psaL2⁻* group. However, the height differences between *psaF2⁻*, *psaJ2⁻* toward WT are lower than those between *psaF2⁻*, *psaJ2⁻* toward *psaI2⁻* and *psaL2⁻* near the FRL absorption region around 710 nm. Also, the curves are further normalized to a peak of 679 nm (**fig. 7B**). The results are similar to **fig. 7A** and have a slight difference in the *psaI2⁻* absorbance spectrum curve. After normalized to a peak point of 679 nm, which is the maximum absorbance peak of Chl *a*, the curve of *psaI2⁻* shift to a higher status and has the same height as FRL-WT of the FRL absorption peak around 710 nm. And for the absorption spectra covering the whole range from 400 – 800 nm of Syn7335 WT, *psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, and *psaL2⁻* are shown in **appendix 1**.

3.4 Room temperature fluorescence spectra of FRL Syn7335 WT, *psaF2*⁻, *psaI2*⁻, *psaJ2*⁻, *psaL2*⁻ cells excited at 440 nm.

As shown in **fig. 8**, the whole cell room temperature (RT) fluorescence spectrum of *psaF2*⁻ has the highest peak at 730 nm, which is the emission from Chl *f*. And the amplitude of the fluorescence level at 730 nm from top to bottom is *psaJ2*⁻, WT, *psaI2*⁻, and the least *psaL2*⁻. The low Chl *f* emission of *psaI2*⁻ and *psaL2*⁻ indicates that knocking out *psaI2* or *psaL2* may affect the PSI structure and energy transfer. The other possibility is that the sites of Chl *f* molecules are located near PsaI2 or PsaL2, and knocking out these two subunits would reduce the peak height of Chl *f* emission. The PSI isolation experiments were further executed to clarify the possibility that losing trimeric PSI would cause a decrease in the emission peak height of Chl *f* molecules of *psaI2*⁻ and *psaL2*⁻.

3.5 Photosystem I isolation of WT and mutants (*psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, *psaL2⁻*)

Fig. 9 shows the PSII and PSI from the FRL Syn7335-WT, and the four mutants (*psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, *psaL2⁻*) were purified. In the first stage of purification, the cells were broken by a mixture of 0.1 and 0.5 mm glass beads, centrifuged down to remove insoluble debris, and loaded into the centrifuge tube for further ultra-high-speed centrifugation.

The first ultra-high-speed centrifugation results show that the supernatant's color contains phycobiliproteins in centrifuge tubes of WT and the four mutants (*psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, *psaL2⁻*) are the same. The color of the supernatant in tubes is bluish, which is the typical color of FRL-type phycobilisomes that loss their phycoerythrin rods.

Further, the thylakoids were solubilized and underwent a second round of ultra-high-speed centrifugation to isolate the photosystem complexes. The isolation results are shown in **fig. 9**. The trimeric structure of PSI can only be found in FRL-WT, *psaF2⁻* and *psaJ2⁻* but not *psaI2⁻* and *psaL2⁻* (Kurashov et al., 2019). This result corresponds with previous whole cell absorption spectra (**fig. 7**) and the RT fluorescence spectra data excited with 440 nm (**fig. 8**). In the spectral data, the FRL absorbance peak and Chl *f* emission of *psaI2⁻* and *psaL2⁻* were vastly decreased compared with FRL-WT, *psaF2⁻*, and *psaJ2⁻*. To summarize, PsaI2 and PsaL2 participate in forming the trimeric structure of PSI, and also losing PsaI2 or PsaL2 would cause a decrease of Chl *f* molecules located nearby (C. J. Gisriel et al., 2022).

3.6 High-Performance Liquid Chromatography (HPLC) of the FRL Syn7335-WT and mutants' (*psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, *psaL2⁻*) cells and their isolated PSI

In **fig. 10** shows the ratios of the three chlorophylls (Chl *a*, Chl *d*, and Chl *f*) in the 100 % stacked bar chart. The percentage of Chl *a*: Chl *d*: Chl *f* is similar between FRL-WT, *psaF2⁻*, and *psaJ2⁻* cells. And for the cells' HPLC results of the FRL Syn7335-WT and mutants, (*psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, *psaL2⁻*) cells are shown in **appendix 2**. In appendix 2, *psaI2⁻* and *psaL2⁻* have lower Chl *f* content than FRL Syn7335-WT, *psaF2⁻* and *psaJ2⁻*. However, the ratio of Chl *a*: Chl *d*: Chl *f* shows a different trend between the trimeric structure PSI of FRL Syn7335-WT, *psaF2⁻*, and *psaJ2⁻*. As in **fig. 11** shown, the *psaJ2⁻* had the lowest Chl *f* molecules ratio between the trimeric structure PSI of FRL Syn7335-WT, *psaF2⁻*, and *psaJ2⁻*. Since each monomer of the FRL Syn7335-WT PSI has 92 chlorophylls, and chlorophyll site at B30 was proposed to be Chl *f* binding site close to PsaJ2 (Christopher J. Gisriel, David A. Flesher, et al., 2022). The Chl *f* content decreased by ~1 % in trimeric PSI of *psaJ2⁻* calculated from the HPLC compared with FRL Syn7335-WT and *psaF2⁻*. Then the HPLC of the PSI trimeric structure can serve as another evidence to support that B30 is a Chl *f* binding site when specification on the mutant of *psaJ2⁻*.

3.7 Absorbance spectra and fluorescence spectra of isolated PSI complexes

As **fig. 12** shown, the absorption spectra of the PSI trimer of FRL Syn7335-WT, *psaF2⁻*, and *psaJ2⁻* are very similar in the far-red light absorbance region (700 – 750 nm). The Chl *a* absorbance peak around 680 nm has a slight difference of *psaF2⁻* which has a blue shift band peaked at 676.5 nm compared with FRL Syn7335-WT and *psaJ2⁻*, which had a peak near 680 nm. And in the RT fluorescence spectra excited at 440 nm shown in **fig. 13**, the blue shift happening in the Chl *f* emission is evident that the trimeric structure of *psaF2⁻* and *psaJ2⁻* has Chl *f* emission peak at 738 nm and 737 nm, respectively. Also, the emission peak height of *psaJ2⁻* is lower than the emission peak of trimeric PSI of FRL Syn7335-WT. As for the 77K fluorescence spectra (**fig. 14**) of the purified trimeric PSI of Syn7335-WT, *psaF2⁻*, and *psaJ2⁻*, the phenomenon of blue-shifted emission peaks can also be found in the trimeric PSI of *psaF2⁻* and *psaJ2⁻* which both having the emission peak of PSI located at 743 nm and have 2 nm blue shift compared with the trimeric PSI of FRL Syn7335-WT.



4. Discussion

4.1 The blue shift of *psaF2⁻* and *psaJ2⁻* under RT and 77K fluorescence spectrum

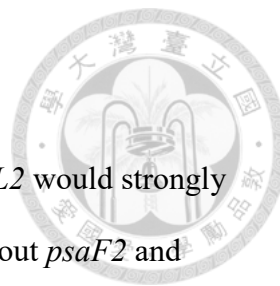
In the fluorescence spectra, the *psaF2⁻* and *psaJ2⁻* have peaks slightly blue-shifted compared with the one in WT (**fig. 13, fig. 14**). And this result corresponds to previous research that simultaneously knockout white light type of *psaF* and *psaJ* in *Synechocystis* PCC 6803 (Jeanjean et al., 2003). The authors proposed that peaks slightly blue-shifted in fluorescence spectra may arise from the knockout of *psaF* and *psaJ*, causing the cells to express *isiA* and *isiB*, similar to iron-deficient stress. This possibility can be ruled out after conducting the MS/MS experiment to identify the protein in *psaF2⁻* and *psaJ2⁻*. Here, the symptom can also be seen when knocking out far-red light type *psaF2* or *psaJ2*, respectively. From this data, we can infer that losing PsaF2 or PsaJ2 would not affect the trimeric structure of PSI but may cause some stress with symptoms similar to iron-deficient stress. The other possibility is that removing PsaF2 or PsaJ2 may cause the removal of A1 and A2 chlorophyll dimer (C. J. Gisriel et al., 2022), that the A1 and A2 chlorophyll dimer may originally contribute to much longer wavelength fluorescence emission. Also, the removal of PsaF2 and PsaJ2 may cause the Chl A38/A39 dimer to monomerize, which could originally offer the ability to absorb longer wavelength energy (C. J. Gisriel et al., 2022). Based on the structural analysis of cryo-EM, the blue shift happening in the absorbance and fluorescence spectrum when losing PsaF2 or PsaJ2 may result from losing the chlorophylls nearby, which may contribute to FRL absorbance or fluorescence emission.

4.2 The Chl *f* binding site specification near PsaJ2

In the HPLC analysis of the trimeric structure of *psaJ2*⁻, the number of Chl *f* molecules was lower compared with FRL Syn7335-WT and the *psaF2*⁻. And in the previous studies of cryo-EM, they found that the sequence homology of the PsaJ2 sidechain is the same as *Fischerella* 7521 and *Halomicronema hongdechloris*, which have the PsaJ2-Tyr sidechain to serve as H-bond to the Chl *f* B30 (C. J. Gisriel et al., 2022). Moreover, the sites B30 and B7 are related by the pseudo C2 symmetry toward the core; it was proposed to be one of the six high-specificity Chl *f* binding sites in FRL Syn7335 PSI (C. J. Gisriel et al., 2022). Combined with the structural studies, our mutagenesis results of HPLC analysis (**fig. 10, fig. 11**) can serve as another evidence that B30 in FRL-PSI is the Chl *f* binding site.

4.3 PSI structure changes in *psaI2⁻* and *psaL2⁻* and the exploration of Chl *f* binding abilities

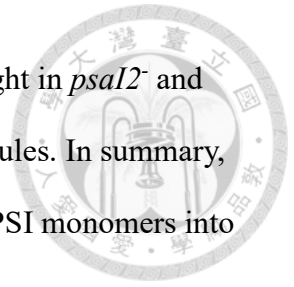
In the absorption spectra and RT fluorescence spectra of the FRL *psaI2⁻* and *psaL2⁻* cells, the FRL absorbance ability, Chl *f* emission peak height of *psaI2⁻* and *psaL2⁻* were strongly decreased (**fig. 7**, **fig. 8**). And in the PSI isolation experiment results, the two mutants of *psaI2⁻* and *psaL2⁻* lost their trimeric structure of PSI. Also, these results can be compared with previous studies that knockout WL-type *psaI* and *psaL* and found that the trimeric structure of PSI is affected (Karapetyan et al., 1999; Schluchter et al., 1996). Furthermore, in the cryo-EM structure of FRL Syn7335-WT PSI structure, it proposed six high-specificity Chl *f* binding sites. The sites of B37 and B38 are very closely related to the region of PsaL2, and B7 is closely related to PsaI2 (Christopher J. Gisriel, David A. Flesher, et al., 2022; Christopher J. Gisriel, Gaozhong Shen, et al., 2022). Based on the structural data of cryo-EM (**fig. 3**), the decrease of FRL absorbance ability (**fig. 7**), Chl *f* emission peak height (**fig. 8**) of *psaI2⁻* and *psaL2⁻* can be attributed not only to the PSI structure and also to the decrease of Chl *f* molecules. The function of PsaI2 and PsaL2 may serve as the bridge to connect each PSI monomers and participate a vital character in the binding of Chl *f* molecules.



5. Conclusion and future works

To summarize the importance of the results, knockout *psaI2* and *psaL2* would strongly affect the growth of the Syn7335 cells under FRL. In contrast, knockout *psaF2* and *psaJ2* would not affect the Syn7335 cells' growth under WL or FRL. Further, *psaL2*⁻ cells have lower FRL absorbance ability, and *psaI2*⁻ and *psaL2*⁻ cells have lower Chl *f* emission peak height detected under RT fluorescence spectra. And for the PSI isolation result, *psaF2*⁻ and *psaJ2* can still form the trimeric structure of PSI, whereas *psaI2*⁻ and *psaL2*⁻ cannot form trimeric structures of PSI and only have one green band located on the upper layer of the sucrose gradient. In the absorption spectra, RT fluorescence spectra, and 77K fluorescence spectra of the trimeric PSI, trimeric PSI purified from *psaF2*⁻ and *psaJ2*⁻ have blue-shifted peaks of Chl *a* absorbance, Chl *f* emission peak, and emission peak of PSI compared with trimeric PSI purified from FRL Syn7335-WT. The blue-shifted phenomenon in *psaF2*⁻ and *psaJ2*⁻ may arise from the loss of PsaF2 and PsaJ2, respectively, or both of PsaF2 and PsaJ2, causing the symptom similar to iron deficient and express *isiA* and *isiB*. And the latter possibility can be ruled out in the future by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to identify protein subunits in *psaF2*⁻ and *psaJ2*⁻. Also, the trimeric PSI of *psaF2*⁻ and *psaJ2*⁻ can be sent to LC-MS/MS to identify the protein in *psaF2*⁻ and *psaJ2*⁻. And for the other possibility is that removing PsaF2 or PsaJ2 may cause loss of A1 and A2 chlorophyll dimer (C. J. Gisriel et al., 2022), which might contribute to much longer wavelength fluorescence emission. Also, the removal of PsaF2 and PsaJ2 may cause the Chl A38/A39 monomerization, which initially served the ability to absorb longer wavelength energy (C. J. Gisriel et al., 2022). This possibility can be further unraveled by sending the trimeric PSI purified from *psaF2*⁻ and *psaJ2*⁻ to identify the structure and chlorophyll molecules binding nearby under cryo-EM.

The decrease of FRL absorbance ability and Chl *f* emission peak height in *psaL2⁻* and *psaL2⁻* can be attributed to the PSI structure and reduced Chl *f* molecules. In summary, PsaI2 and PsaL2 can serve as bridges connecting three independent PSI monomers into PSI trimer and also participate in binding with Chl *f* molecules.



Figures

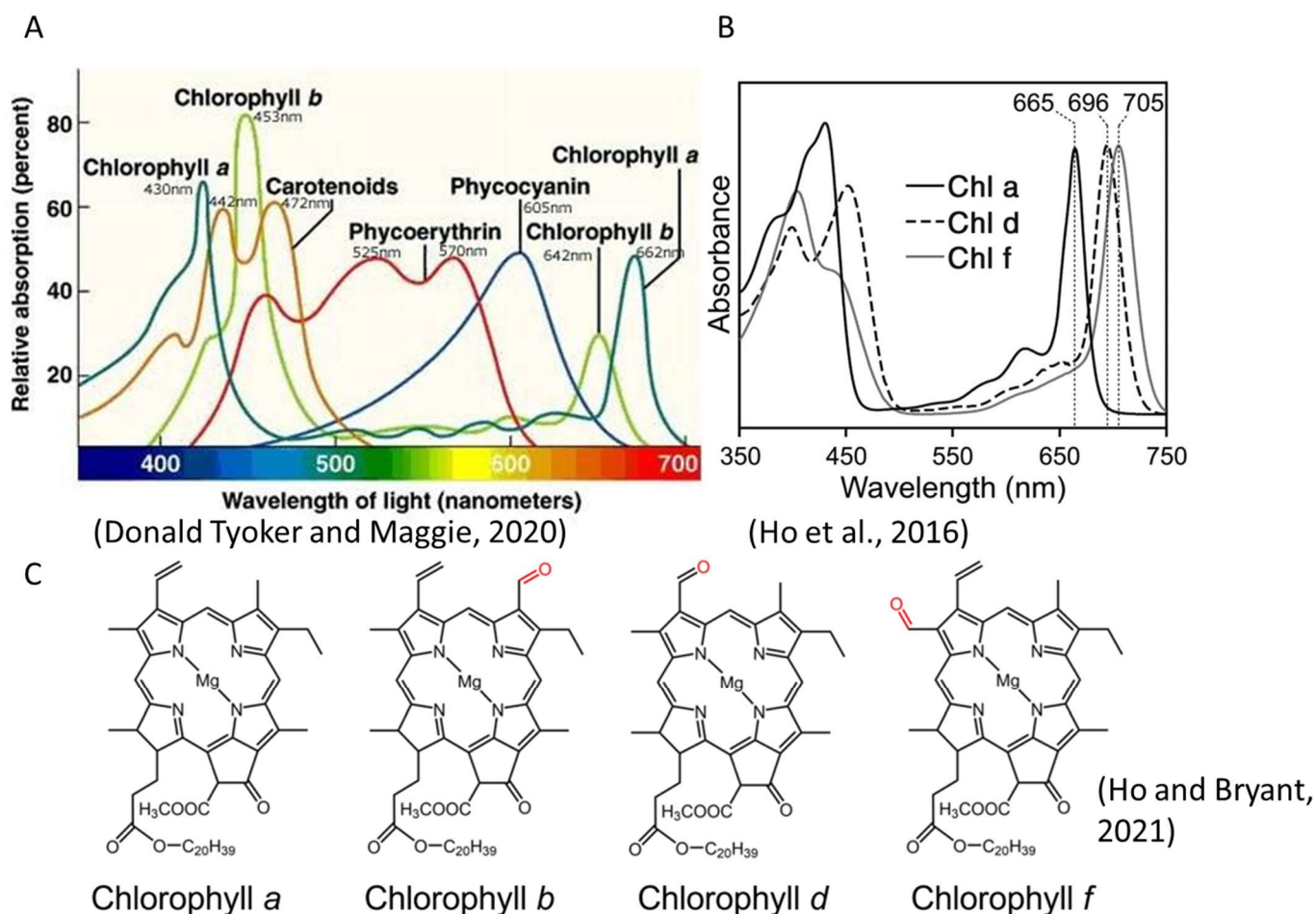


Fig. 1 Protein and pigments absorption spectra and the structure of chlorophylls

Fig. 1A is modified from (Donald Tyoker & Maggie, 2020); the central absorbance peak of chlorophyll *a*, chlorophyll *b*, and carotenoids is mainly located in the blue, purple, red, and orange regions. In contrast, phycoerythrin and phycocyanin have absorbance peaks in green and yellow areas. In **fig. 1B**, modified from (Ho et al., 2016), the absorbance peak of chlorophyll *d* and chlorophyll *f* is located at 696 nm and 705 nm, respectively, which is more closely to the far-red region compared with chlorophyll *a* with a center at 665 nm. The chemical structures of Chl *a*, Chl *b*, Chl *d*, and Chl *f* are shown in **fig. 1C** (modified from (Ho & Bryant, 2021)), and the different sites are marked with red.

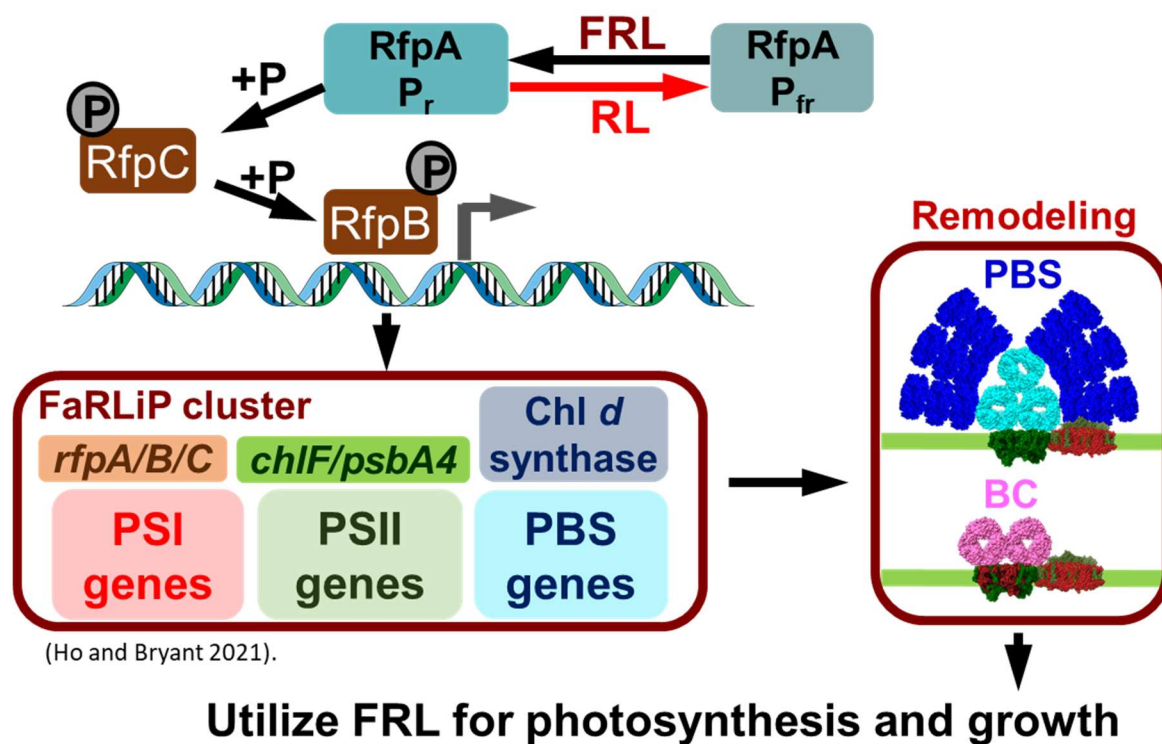


Fig. 2 Regulation mechanism of FaRLiP.

Fig. 2 is modified from (Ho & Bryant, 2021). Receiving the light of FRL, the P_{fr} form of RfpA would turn into P_r form and then phosphorylate downstream RfpC. Further, the phosphorylated RfpC would phosphorylate RfpB, open the downstream FaRLiP gene cluster, and express 20 genes to remodel PSII, PSI, and phycobilisomes.

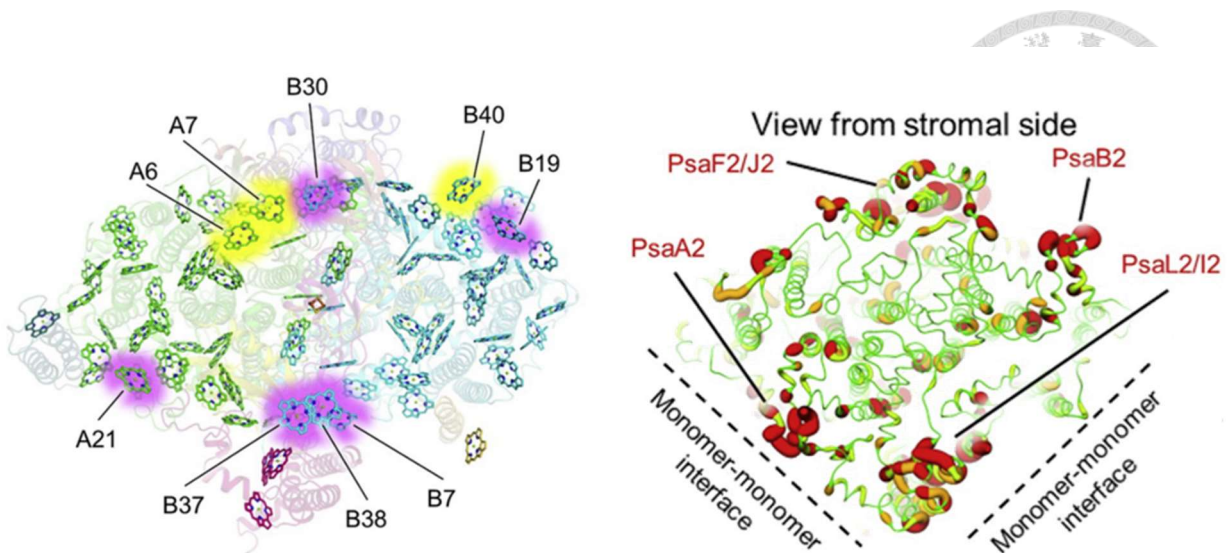


Fig. 3 Possible Chl *f* binding sites based on the cryogenic electron microscopy (cryo-EM) data from (C. J. Gisriel et al., 2022; C. J. Gisriel et al., 2020)

Fig. 3 is modified from (C. J. Gisriel et al., 2022). In the structural data identified under cryo-EM, several possible Chl *f* molecules binding sites were proposed based on visual inspection of the electrostatic potential map to the C2 moieties and cone scan analysis. The chlorophyll sites with a pink glow indicate the higher specificity of Chl *f* binding sites. In contrast, the chlorophyll sites with a yellow glow show the lower specificity Chl *f* binding sites. Here four subunits (*psaF2*, *psaI2*, *psaJ2*, and *psaL2*) near the possible binding sites were chosen as the target gene for further mutagenesis study.

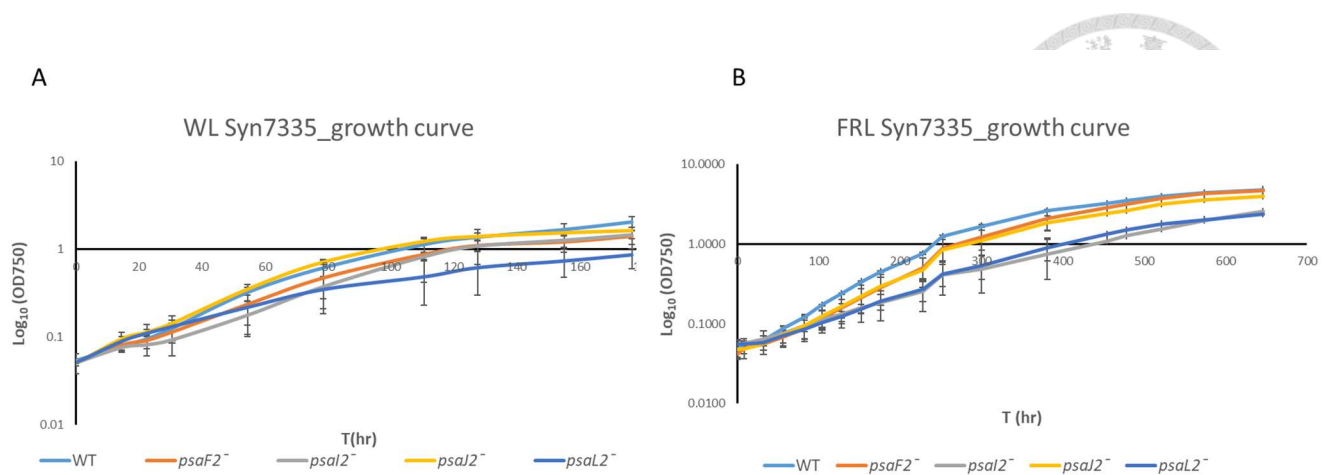


Fig. 5 Growth curve of Syn7335 WT and mutants under WL and FRL.

The WT and mutants' (*psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, and *psaL2⁻*) growth curves of Syn7335 were grown under a light intensity of $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ visible light and far-red light (LED centered at 730 nm) of light intensity of $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. In **Fig. 5A**, Syn7335-WT and the mutants (*psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, and *psaL2⁻*) growth curves are similar when grown under WL. Whereas, in FRL (**fig. 5B**), *psaI2⁻* and *psaL2⁻* grew slower compared with Syn7335-WT, *psaF2⁻*, and *psaJ2⁻*.

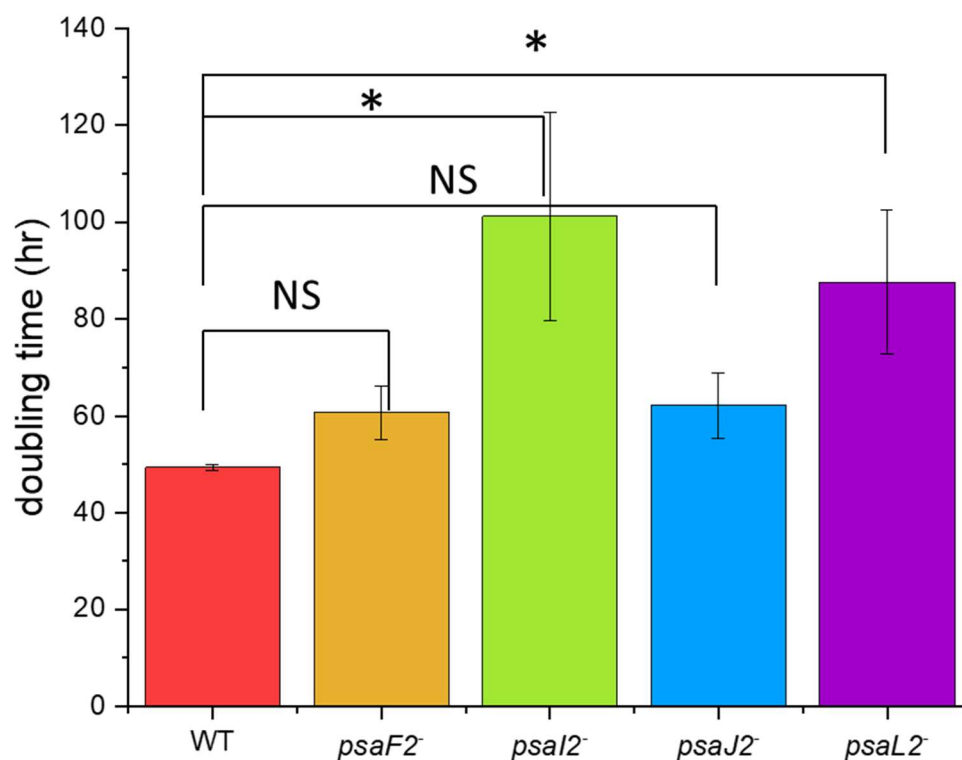


Fig. 6 The doubling time of the FRL Syn7335-WT and the four mutants (*psaF2*⁻, *psaI2*⁻, *psaJ2*⁻, and *psaL2*⁻) cells.

(<https://www.statskingdom.com/kruskal-wallis-calculator.html>)

The p-value was calculated with the Kruskal-Wallis calculator with a significance level: *p<0.05; NS: no significant difference. In **fig. 6**, the doubling time of Syn7335-WT has the lowest doubling time when grown under FRL but has no significant difference between *psaF2*⁻ and *psaJ2*⁻. However, the doubling time of *psaI2*⁻ and *psaL2*⁻ have significantly longer doubling times compared with Syn7335-WT under FRL.

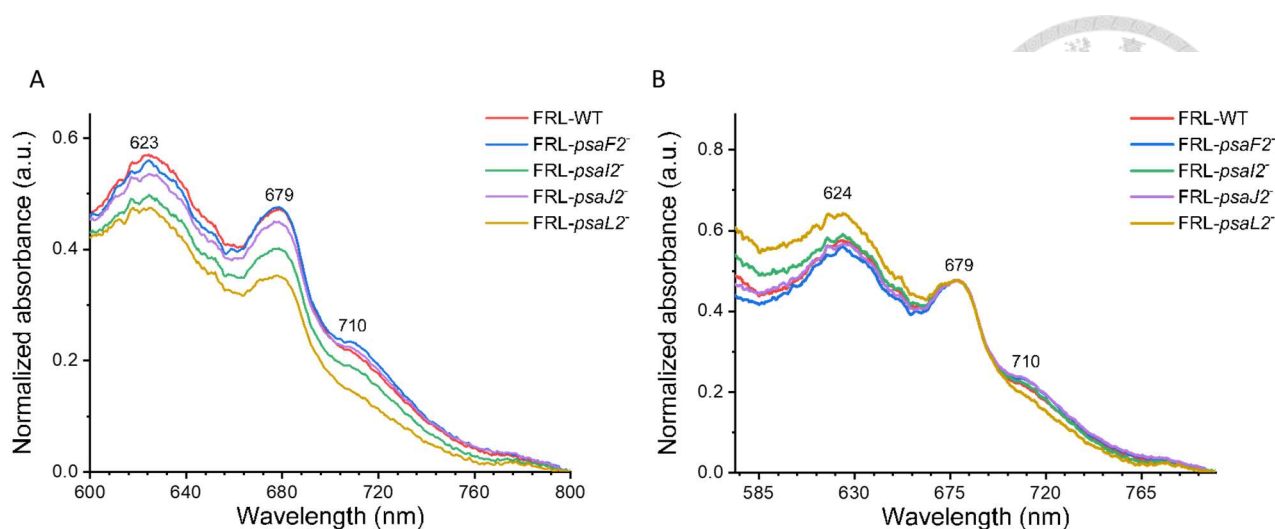


Fig. 7 Syn7335 WT and mutants' (*psaF2*⁻, *psaI2*⁻, *psaJ2*⁻, and *psaL2*⁻) absorption spectra

In the absorption spectra (**fig. 7A**), the FRL absorbance peak (700 – 750 nm) of *psaI2*⁻ and *psaL2*⁻ was lower than others. And in **fig. 7B**, the absorption curves were normalized at the picked point at 680 nm. The *psaF2*⁻ and *psaJ2*⁻ cells have the highest FRL absorption peak height, FRL Syn7335-WT, *psaI2*⁻ cells ranked second, and *psaL2*⁻ has the lowest FRL absorbance ability.

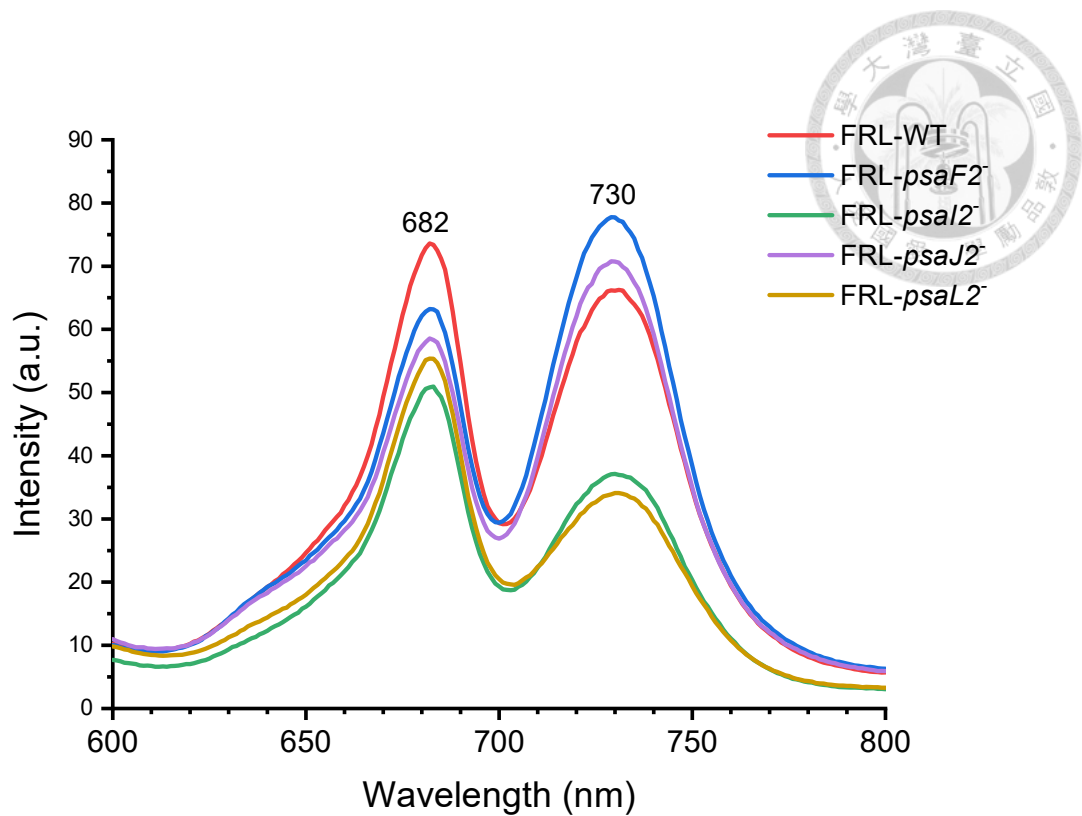


Fig. 8 The RT fluorescence spectrum of the FRL Syn7335-WT and the four mutants (*psaF2*⁻, *psaI2*⁻, *psaJ2*⁻, *psaL2*⁻) cells

As shown in **fig. 8**, *psaF2*⁻ and *psaJ2*⁻ have the highest Chl *f* emission peak at around 730 nm, slightly higher than the Chl *f* emission peak of FRL Syn7335-WT. Otherwise, *psaI2*⁻ and *psaL2*⁻ have the lowest Chl *f* emission peak height at around 730 nm.

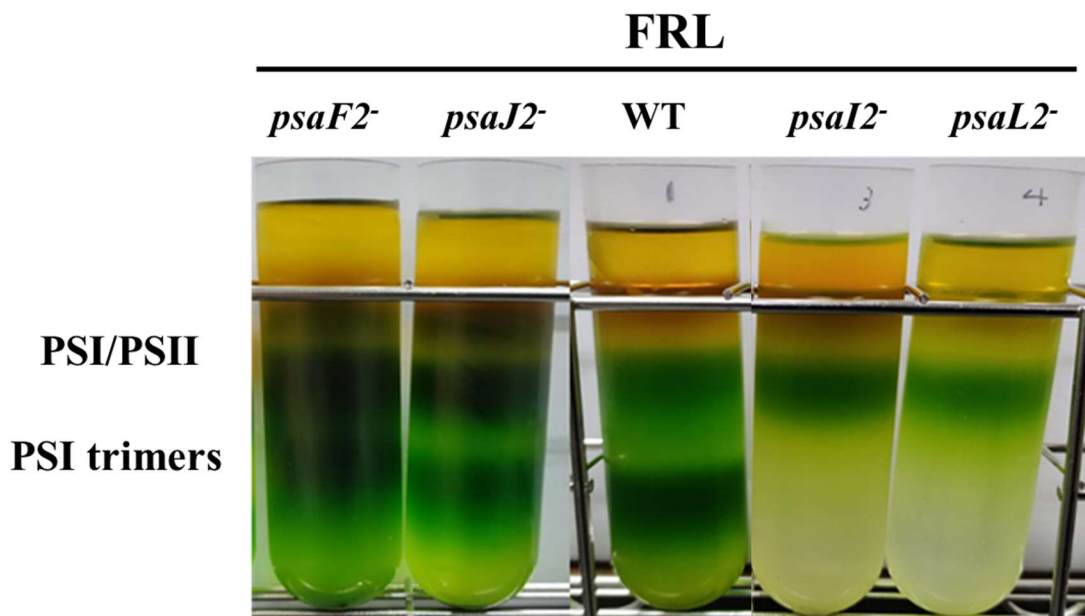


Fig. 9 Isolation of PSI complexes from Syn7335-WT and *psaF2⁻*, *psaI2⁻*, *psaJ2⁻*, *psaL2⁻* cells

In **fig. 9**, the monomeric and trimeric fractions containing PSI and PSII were isolated by ultracentrifugation. In the isolated result, the trimeric structure of PSI can only be found in FRL-WT, *psaF2⁻*, and *psaJ2⁻* but cannot be found in FRL *psaI2⁻* and *psaL2⁻*.

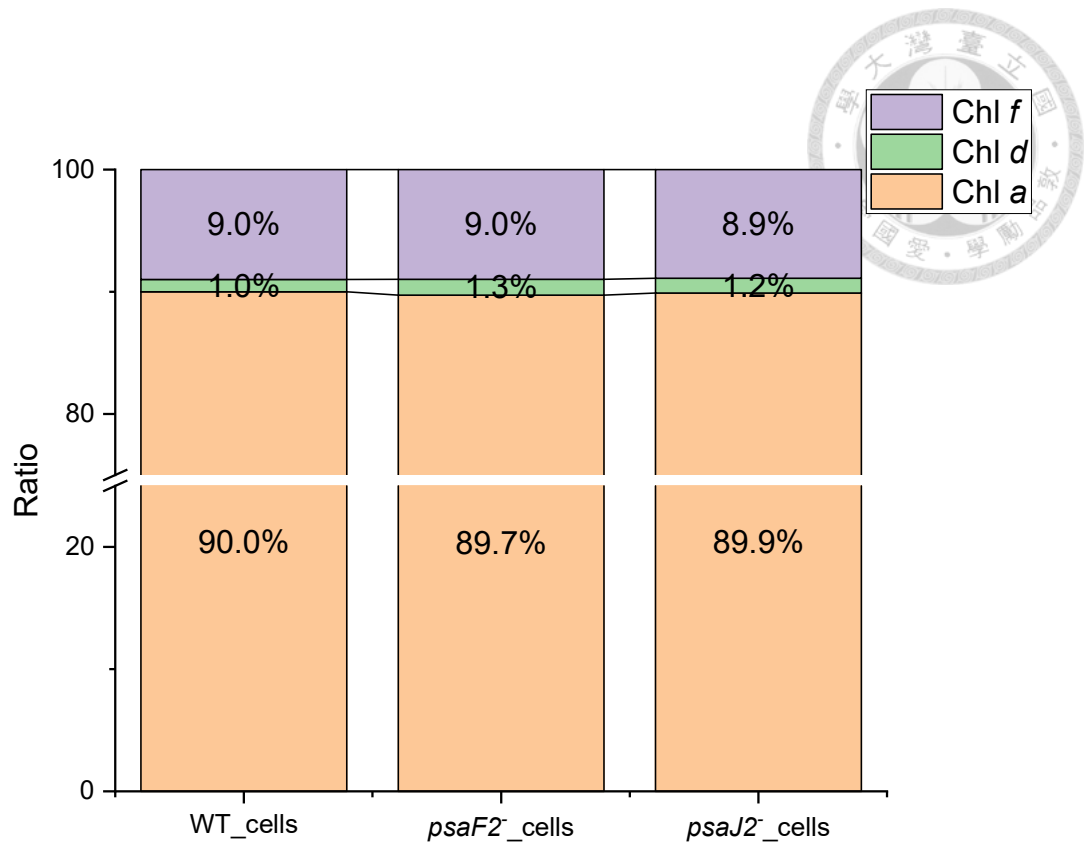


Fig. 10 The chlorophyll ratio of Chl *a*: Chl *d*: Chl *f* in the 100 % stacked bar chart of FRL Syn7335-WT, *psaF2*⁻, *psaJ2*⁻ cells

The Chl *f* ratios do not change much along with *psaF2*⁻, *psaJ2*⁻ and WT in cells. Here the Chl *d* molecules were produced when extracted the pigments since the Chl *a* molecules would spontaneously turn into Chl *d* molecules non-enzymatically (Fukusumi et al., 2012).

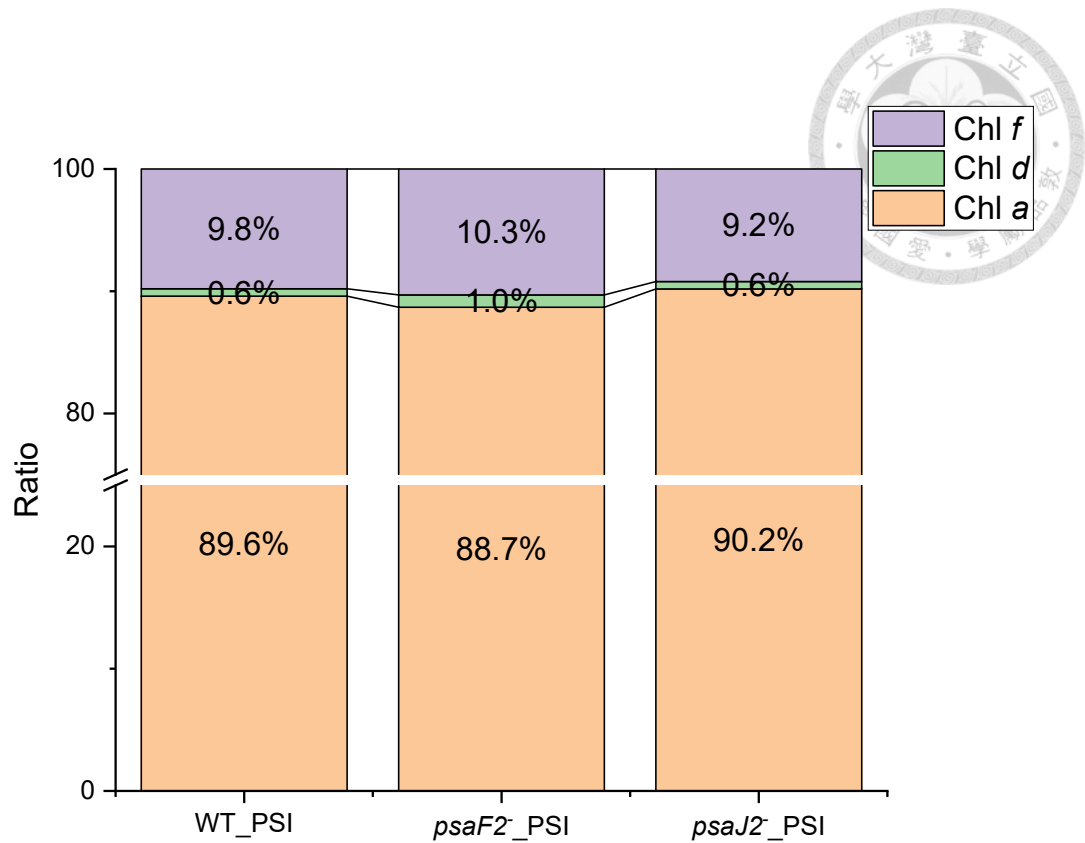


Fig. 11 The chlorophyll ratio of Chl *a*: Chl *d*: Chl *f* in the 100 % stacked bar chart of the trimeric PSI purified from FRL Syn7335-WT, *psaF2*⁻, *psaJ2*⁻

In **fig. 11** shows lower chlorophyll *f* in the *psaJ2*⁻ PSI trimer compared with the trimeric PSI purified from FRL Syn7335-WT and *psaF2*⁻. Here the Chl *d* molecules were produced when extracted the pigments since the Chl *a* molecules would spontaneously turn into Chl *d* molecules non-enzymatically (Fukusumi et al., 2012).

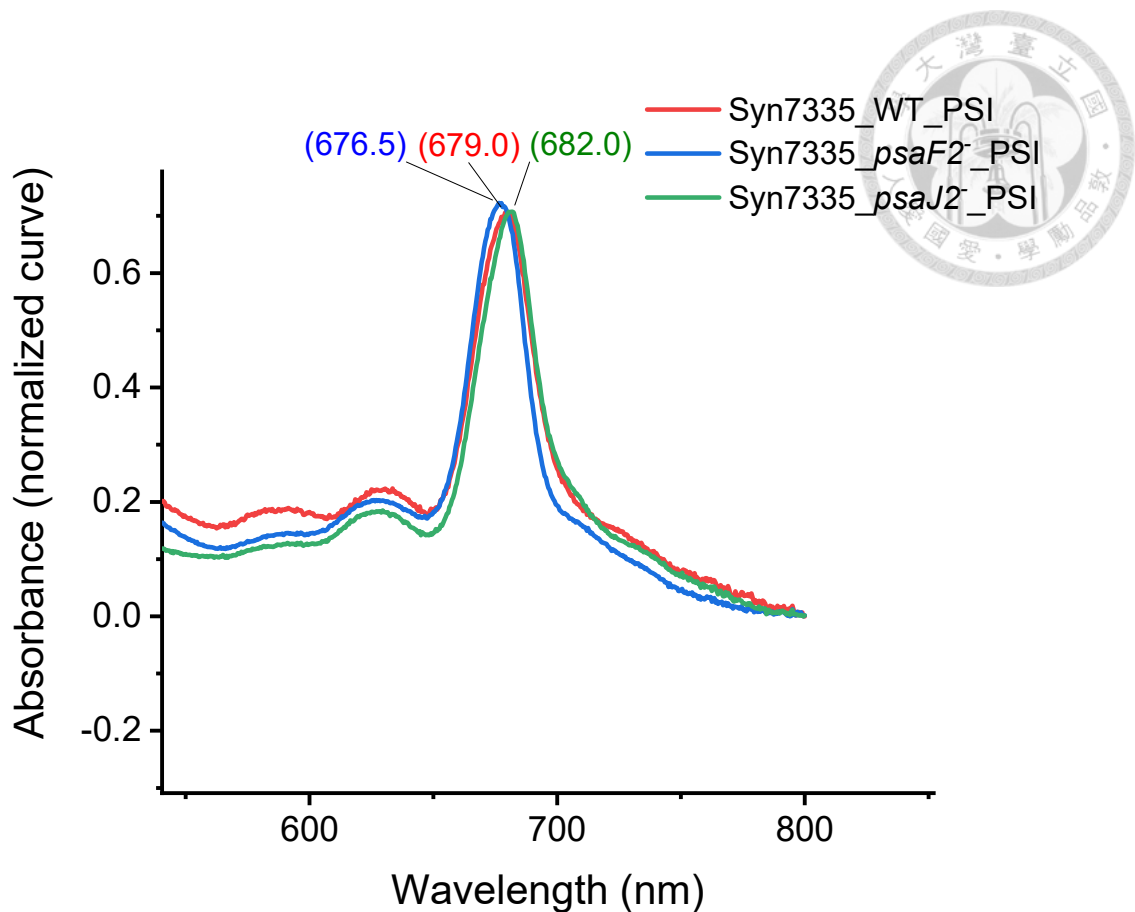


Fig. 12 Absorbance spectra of Syn7335 WT, *psaF2*, *psaJ2* PSI trimer.

The absorbance spectra curves of the trimeric PSI purified from FRL Syn7335-WT, *psaF2*, and *psaJ2* are shown in **fig. 12**. The absorbance spectra shown in **fig. 12** are normalized from 0 – 1 and then normalized to the peak point at 680 nm. In the absorbance spectra, the PSI trimer of *psaF2* has slightly blue shift peaks at around 680 nm compared with the PSI trimer of FRL Syn7335-WT.

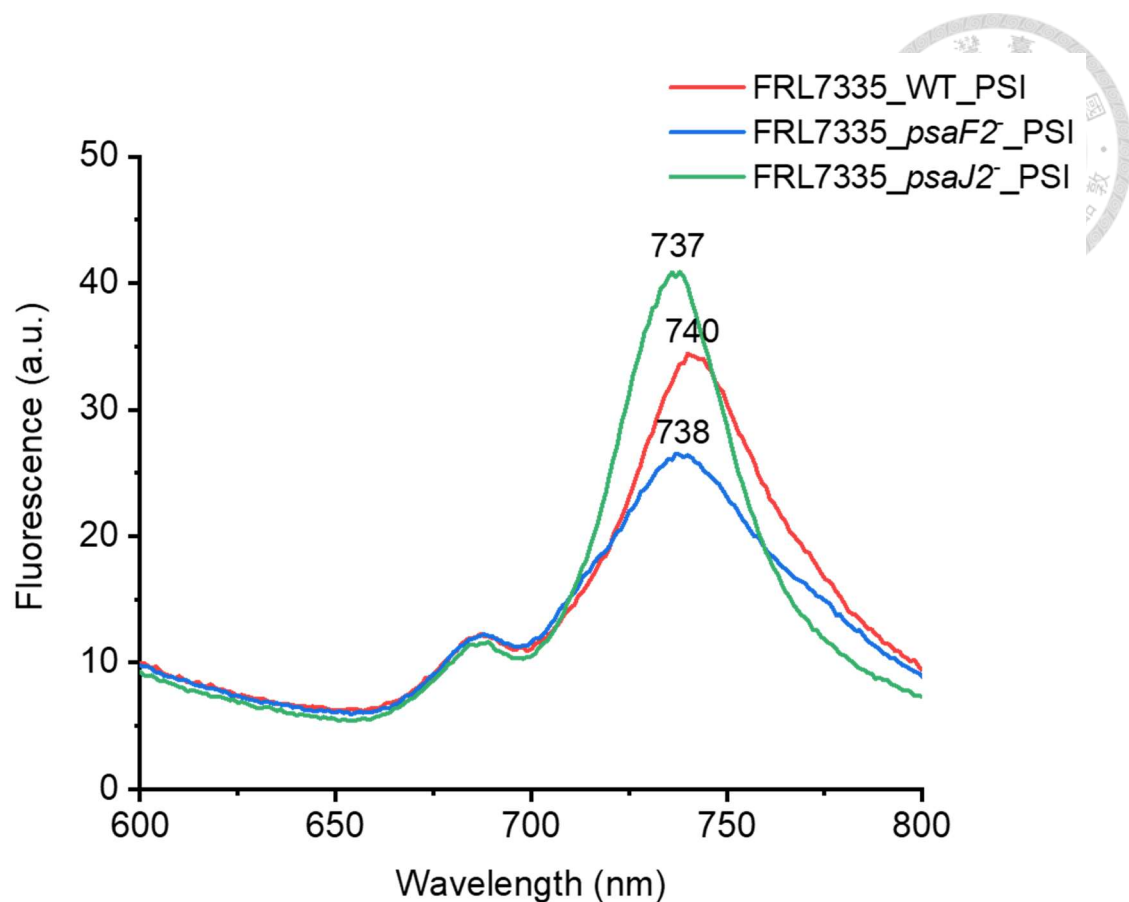


Fig. 13 RT fluorescence spectra of Syn7335 WT, *psaF2⁻*, *psaJ2⁻* PSI trimer.

In **fig. 13**, the PSI trimer of *psaF2⁻* has the lowest Chl *f* emission peak height compared with the PSI trimer of FRL Syn7335-WT and *psaJ2⁻*. Also, the Chl *f* emission peak of *psaF2⁻* and *psaJ2⁻* slightly blue shift to 738 nm and 737 nm, respectively, which is different from the Chl *f* emission peak of the FRL Syn7335-WT PSI trimer located at 740 nm.

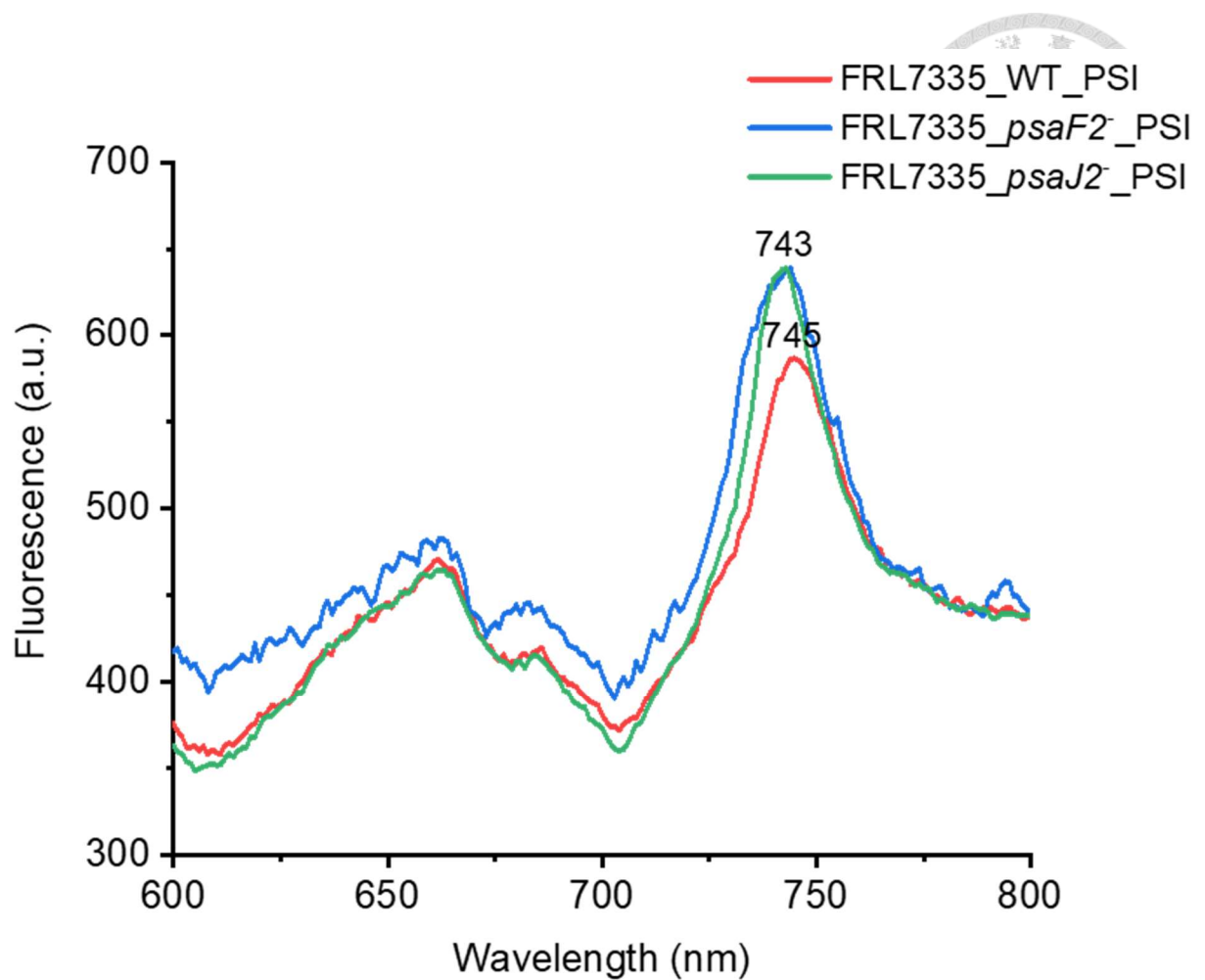


Fig. 14 77K fluorescence spectrum of Syn7335 WT, *psaF2*⁻, *psaJ2*⁻ PSI trimer.

In **fig. 14**, the PSI trimer of *psaF2*⁻ and *psaJ2*⁻ have a blue shift of the PSI emission peak located at 743 nm, which has a two nanometers shift compared with the PSI emission peak of FRL Syn7335-WT PSI located at 745 nm.

Tables

Table 1 Medium solution of ASN-III liquid and plate

ASNIII liquid medium & 1.75% plate

	1L
NaCl	25.0 g
MgSO ₄ ·7H ₂ O	3.5 g
MgCl ₂ ·6H ₂ O	2.0 g
NaNO ₃	0.75 g
CaCl ₂ ·2H ₂ O	0.5 g
KCl	0.5 g
Na ₂ CO ₃	0.02 g
Citric acid	1 ml from stock (3.0 mg)
Ferric ammonium citrate	1 ml from stock (3.0 mg)
A-5 trace minerals	1.0 ml
EDTA	0.167 ml from 3g/L stock (0.5 mg)
Vitamin B ₁₂ (Add after autoclave)	1 ml from stock (4.0 mg)
K ₂ HPO ₄ (Add after autoclave)	1 ml from stock (0.064 g)
1 M Tris-HCl, pH 8 (Add after autoclave)	10 ml
Triple-washed Bacto agar	17.5 g
Distilled water to	1.0 L

Final pH ~8.6 at room temperature

Trace metal mix A5:

H_3BO_3	2.86 g
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1.81 g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.222 g
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.39 g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.079 g
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	49.4 mg
Distilled water	1.0 L



Table 2 Primers used in this study

primer name	Size (base)	Sequence (5' to 3')	description
(KC1)-7335-pasL2-3kb-upstream-F	60	TTCGCCCAGCTTCTGTATGGA ACGGGCATGGGAAAATGCAG TCAGATGTCTGGGGAGCGG	for amplification of the 3 kb upstream region of <i>psaL2</i> in Syn7335
(KC2)-7335-pasL2-3kb-upstream-R	59	CCTTGCGCCCTGAGTGCTTGC GGCAGCGTGGGATCCAGAAA TAACCTCACTAGAATTGC	for amplification of the 3 kb upstream region of <i>psaL2</i> in Syn7335
(KC3)-7335-pasL2-3kb-downstream-F	59	ATCTCATGCTGGAGTTCTTCG CCCACCACTAGTTTTTGTCT TCTTTTGTCTTCATAG	for amplification of the 3 kb downstream region of <i>psaL2</i> in Syn7335
(KC4)-7335-pasL2-3kb-downstream-R	60	CGCAACGTTGTTGCCATTGCT GCAGGCATGGCCTATCTAGCT GGCTCGATCTCAAGTCTC	for amplification of the 3 kb downstream region of <i>psaL2</i> in Syn7335
(KC5)-pasL2_km_seqencing_F	22	AGAGTACTGCTGGGGCAAGC CA	colony PCR primer for conjugated p
(KC6)-pasL2_km_seqencing_R	24	GGCGATCGCGAATATTGGGAA GGG	colony PCR primer for conjugated plasmid pRL277-7335-psaL2-Km
(KC7)-pRL277_7335psaL2Km_seq_F1	22	CGACTCACCCCGTCGCCTATG A	sequencing primer for conjugated plasmid 7335-pRL277- <i>psaL2</i> -km
(KC8)-pRL277_7335psaL2Km_seq_F2	22	AGGGAACTGGGGAGCCTATG CG	sequencing primer for conjugated plasmid 7335-pRL277- <i>psaL2</i> -km

(KC9)- pRL277_7335psaL2Km_se q_F3	25	GCTCTCTATGGCATTAGCTCG CTGC	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaL2</i> -km
(KC10)- pRL277_7335psaL2Km_se q_F4	22	TACTGGCAGCAGGTTCCCGT GA	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaL2</i> -km
(KC11)- pRL277_7335psaL2Km_se q_F5	22	CTGCTCTGATGCCGCCGTGTT C	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaL2</i> -km
(KC12)- pRL277_7335psaL2Km_se q_F6	22	TTTCGATTCCACCGCCGCCTT C	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaL2</i> -km
(KC13)- pRL277_7335psaL2Km_se q_F7	22	GCTAACTTGCTTTGCGGCGCA G	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaL2</i> -km
(KC14)- pRL277_7335psaL2Km_se q_F8	22	CCTGCTCTTGAACGCCGCTCT T	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaL2</i> -km
(KC15)- pRL277_7335psaL2Km_se q_F9	22	TGAAAGGGCTGCGTCGAAAG CT	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaL2</i> -km
(KC16)-7335-pRL277- psaL2-km-3kb-upstream-F	59	TCGCCCAGCTTCTGTATGGAA CGGGCATGGCGTCCCATTG GCCATCTTGCCATTATC	for amplification of the 3 kb upstream region of <i>psaL2</i> in Syn7335
(KC17)-7335-pRL277- psaL2-km-3kb-upstream-R	59	CCCTGAGTGCTTGCGGCAGC GTGGGATCCCTCATCAAATCT	for amplification of the 3 kb upstream region of <i>psaL2</i> in

		CCTATCCAATCGTAAGGG	Syn7335
(KC18)-7335-pRL277- psaI2-km-3kb- downstream-F	59	CATGCTGGAGTTCTTCGCCCA CCACTAGTCGACTCAACCGC AACTTGATATTCCGTGTC	for amplification of the 3 kb downstream region of <i>psaI2</i> in Syn7335
(KC19)-7335-pRL277- psaI2-km-3kb- downstream-R	59	GCAACGTTGTTGCCATTGCTG CAGGCATGGCGGCATCGAGA TATCTAATAGCAGCAAAT	for amplification of the 3 kb downstream region of <i>psaI2</i> in Syn7335
(KC20)-7335-pRL277- psaI2-km-3kb-upstream-F- automatic_generate	40	TTCTGTATGGAACGGGCATGG CGTCCCATTGTTGGCCATCT	for amplification of the 3 kb upstream region of <i>psaI2</i> in Syn7335
(KC21)-7335-pRL277- psaI2-km-3kb-upstream-R- automatic_generate	44	CTTGCGGCAGCGTGGGATCC CTCATCAAATCTCCTATCCAAT CG	for amplification of the 3 kb upstream region of <i>psaI2</i> in Syn7335
(KC22)-7335-pRL277- psaI2-km-3kb- downstream-F- automatic_generate	40	GTTCTTCGCCACCACTAGTC GACTCAACCGCAACTTGAT	for amplification of the 3 kb downstream region of <i>psaI2</i> in Syn7335
(KC23)-7335-pRL277- psaI2-km-3kb- downstream-R- automatic_generate	40	TTGCCATTGCTGCAGGCATGG CGGCATCGAGATATCTAAT	for amplification of the 3 kb downstream region of <i>psaI2</i> in Syn7335
(KC24)-7335-pRL277- psaI2- km_km_sequencing_F	22	TTGATGGGCTCATTAGGCGGC G	colony PCR primer for conjugated plasmid pRL277- 7335- <i>psaI2</i> -Km

(KC25)-7335-pRL277- psaI2- km_km_sequencing_R	22	CGGTGAGGAAGCGCAGGGA ACT	colony PCR primer for conjugated plasmid pRL277- 7335- <i>psaI2</i> -Km
(KC26)-7335-pRL277- psaI2- km_colony_PCR_primer_F	22	TTGATGGGCTCATTAGGCGGC G	colony PCR primer for conjugated plasmid pRL277- 7335- <i>psaI2</i> -Km
(KC27)-7335-pRL277- psaI2- km_colony_PCR_primer_ R	22	CGGTGAGGAAGCGCAGGGA ACT	colony PCR primer for conjugated plasmid pRL277- 7335- <i>psaI2</i> -Km
(KC28)- pRL277_7335psaI2Km_se q_F2	22	ATTCCTGAATCGCGCGGACA GC	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaI2</i> -km
(KC29)- pRL277_7335psaI2Km_se q_F3	22	CGCCTTTGGTAGCCCGGACA AA	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaI2</i> -km
(KC30)- pRL277_7335psaI2Km_se q_F4	22	CTATCGATTGTGCAGGCGCGG T	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaI2</i> -km
(KC31)- pRL277_7335psaI2Km_se q_F5	22	CGACTGCGTCCGCTCCGTAA AA	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaI2</i> -km
(KC32)- pRL277_7335psaI2Km_se q_F6	22	GGGCGCCCGGTTCTTTTGTGTC A	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaI2</i> -km
(KC33)-	22	TTTCGATTCCACCGCCGCCTT	sequencing primer for

pRL277_7335psaI2Km_se q_F7		C	conjugated plasmid 7335- pRL277- <i>psaI2</i> -km
(KC34)- pRL277_7335psaI2Km_se q_F8	22	TCGACCACCTCTACCGTGACC A	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaI2</i> -km
(KC35)- pRL277_7335psaI2Km_se q_F9	23	TGTCAGCAACTACGTGCCCA AGA	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaI2</i> -km
(KC36)- pRL277_7335psaI2Km_se q_F10	22	CGCACAGTTTGACAGGGGTG CT	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaI2</i> -km
(KC61)-7335- <i>psaF2</i> - upstream-3kb-F	59	TCGCCCAGCTTCTGTATGGAA CGGGCATGGGCTGCATTTGCA ACCGCGATATCGGCCTA	for amplification of the 3 kb upstream region of <i>psaF2</i> in Syn7335
(KC62)-7335- <i>psaF2</i> - upstream-3kb-R	59	GAGTGCTTGCGGCAGCGTGG GATCCAGCTATGTTTTCTAAT AAACGAATAAATCTTGG	for amplification of the 3 kb upstream region of <i>psaF2</i> in Syn7335
(KC63)-7335- <i>psaF2</i> - downstream-3kb-F	59	CATGCTGGAGTTCTTCGCCCA CCACTAGTCAGCCATCTCGAC CACCTCTACCGTGACCA	for amplification of the 3 kb downstream region of <i>psaF2</i> in Syn7335
(KC64)-7335- <i>psaF2</i> - downstream-3kb-R	59	GCAACGTTGTTGCCATTGCTG CAGGCATGGCAGTGCAACTT TAGCTTCACTGCTTAACA	for amplification of the 3 kb downstream region of <i>psaF2</i> in Syn7335
(KC65)-7335- <i>psaJ2</i> - upstream-3kb-F	59	TCGCCCAGCTTCTGTATGGAA CGGGCATGGTTAGGCTGGCA CCTAGCCTGCCTTGGTGT	for amplification of the 3 kb upstream region of <i>psaJ2</i> in Syn7335

(KC66)-7335-psaJ2-upstream-3kb-R	59	CCCTGAGTGCTTGCGGCAGC GTGGGATCCGTGGGTCTCCA GACAGTAGAGTTGGTCAC	for amplification of the 3 kb upstream region of <i>psaJ2</i> in Syn7335
(KC67)-7335-psaJ2-downstream-3kb-F	59	CATGCTGGAGTTCTTCGCCCA CCACTAGTGCTAGCCCAAAG TCGTATCAATAGCTACGA	for amplification of the 3 kb downstream region of <i>psaJ2</i> in Syn7335
(KC68)-7335-psaJ2-downstream-3kb-R	59	GCAACGTTGTTGCCATTGCTG CAGGCATGGCTGGACAATCT CTAGCTCCCGATCACCAA	for amplification of the 3 kb downstream region of <i>psaJ2</i> in Syn7335
(KC105)-7335-pRL277-psaL2-km-mut_seq_p	22	TCTGGGACCCTCAGTTTGGG CC	sequencing primer of 7335-pRL277- <i>psaL2</i> -km conjugants
(KC106)-pRL277-colony-PCR-primer-set1-F	22	CTACACGACGGGGAGTCAGG CA	colony PCR primer to ensure backbone is not inserted into Syn7335
(KC107)-pRL277-colony-PCR-primer-set1-R	22	ACGTGCAGCTTTCCTTCAGG C	colony PCR primer to ensure backbone is not inserted into Syn7335
(KC108)-pRL277-colony-PCR-primer-set2-F	23	CCCGCGCGATTACTTTTCGA CC	colony PCR primer to ensure backbone is not inserted into Syn7335
(KC109)-pRL277-colony-PCR-primer-set2-R	22	ACGTGCAGCTTTCCTTCAGG C	colony PCR primer to ensure backbone is not inserted into Syn7335
(KC110)-7335-pRL277-psaL2-km-mut_seq_p_R	22	AATCCTGTCCAGCCTGGGCCT T	sequencing primer of 7335-pRL277- <i>psaL2</i> -km

			conjugants
(KC113)-7335-psaF2-colony-PCR-set1-F	22	GGCTCATTAGGCGGCGCAATC T	colony PCR primer for conjugated plasmid pRL277-7335- <i>psaF2</i> -Km
(KC114)-7335-psaF2/J2-colony-PCR-set1-R	22	GCAGTCCTTCCCACTCGAGG GT	colony PCR primer for conjugated plasmid pRL277-7335- <i>psaF2/psaJ2</i> -Km
(KC115)-7335-psaJ2-colony-PCR-set1-F	22	GCCTGCTGTTGCTGCCTCAGA A	colony PCR primer for conjugated plasmid pRL277-7335- <i>psaJ2</i> -Km
(KC116)-pRL277-7335-psaF2_km_seq_F1	22	TGGCGCATCACCATTGCGA T	sequencing primer for conjugated plasmid 7335-pRL277- <i>psaF2</i> -km
(KC117)-pRL277-7335-psaF2_km_seq_F2	22	TGCGATCGCACTTGGTCTACA TGT	sequencing primer for conjugated plasmid 7335-pRL277- <i>psaF2</i> -km
(KC118)-pRL277-7335-psaF2_km_seq_F3	22	ACAAACTTTCTTTGCGATCGC T	sequencing primer for conjugated plasmid 7335-pRL277- <i>psaF2</i> -km
(KC119)-pRL277-7335-psaF2_km_seq_F4	22	TCTGCCCTTCCCAATATTCGC GA	sequencing primer for conjugated plasmid 7335-pRL277- <i>psaF2</i> -km
(KC120)-pRL277-7335-psaF2_km_seq_F5	22	CCTGAATGAACTGCAGGACG AGGC	sequencing primer for conjugated plasmid 7335-pRL277- <i>psaF2</i> -km
(KC121)-pRL277-7335-	22	TCGACCACCTCTACCGTGACC	sequencing primer for

psaF2_km_seq_F6		A	conjugated plasmid 7335- pRL277- <i>psaF2</i> -km
(KC122)-pRL277-7335- psaF2_km_seq_F7	22	ACACCTTGCCTGTGCTTTTCG T	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaF2</i> -km
(KC123)-pRL277-7335- psaF2_km_seq_F8	22	GACGATCAGGCCCGCTTGGA TG	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaF2</i> -km
(KC124)-pRL277-7335- psaF2_km_seq_F9	22	TGTCAAACGGTGCCTCGGTC AC	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaF2</i> -km
(KC125)-pRL277-7335- psaJ2_km_seq_F1	22	CGCCTTTGGTAGCCCGGACA AA	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaJ2</i> -km
(KC126)-pRL277-7335- psaJ2_km_seq_F2	22	GGTTGGTGGGCCTGGTTCAC TT	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaJ2</i> -km
(KC127)-pRL277-7335- psaJ2_km_seq_F3	22	CGATAGCATTCTTCGAGGCGC TGT	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaJ2</i> -km
(KC128)-pRL277-7335- psaJ2_km_seq_F4	22	GCTGGTCTGGCCGGGCTTATC T	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaJ2</i> -km
(KC129)-pRL277-7335- psaJ2_km_seq_F5	22	CCTGAATGAACTGCAGGACG AGGC	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaJ2</i> -km

(KC130)-pRL277-7335- psaJ2_km_seq_F6	22	CGAGAAGGCTTACCGCTGGC TG	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaJ2</i> -km
(KC131)-pRL277-7335- psaJ2_km_seq_F7	22	GGGAAGGACTGCGGCTAGAG GT	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaJ2</i> -km
(KC132)-pRL277-7335- psaJ2_km_seq_F8	22	AGAGTCTTTGGTAGCGATGCT A	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaJ2</i> -km
(KC133)-pRL277-7335- psaJ2_km_seq_F9	22	CGCCACCGCTAATAGCCCTAC C	sequencing primer for conjugated plasmid 7335- pRL277- <i>psaJ2</i> -km

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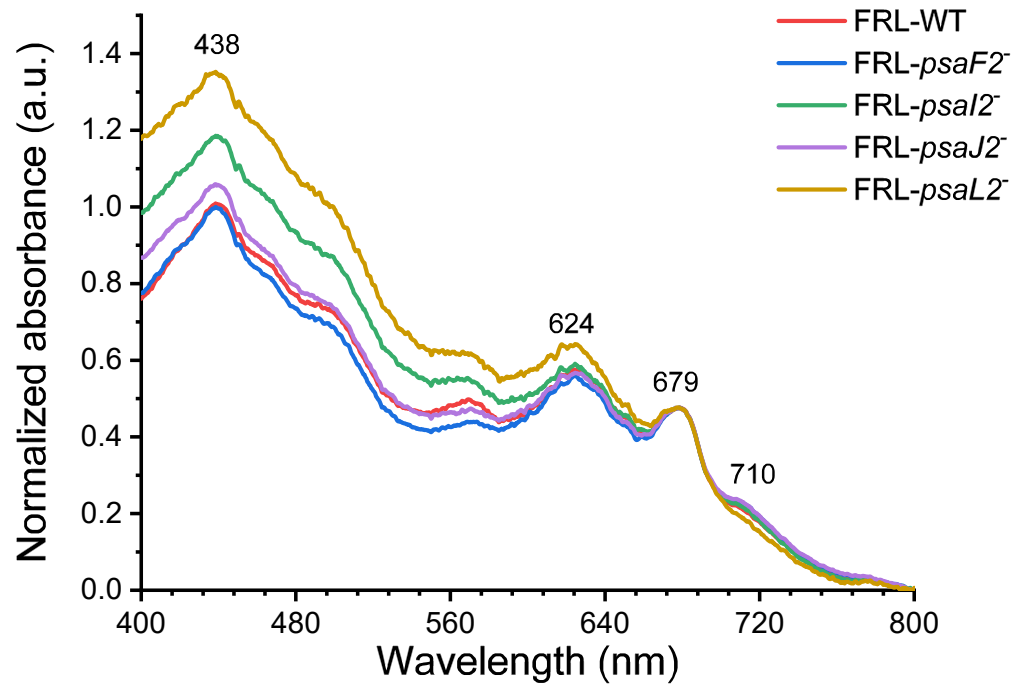
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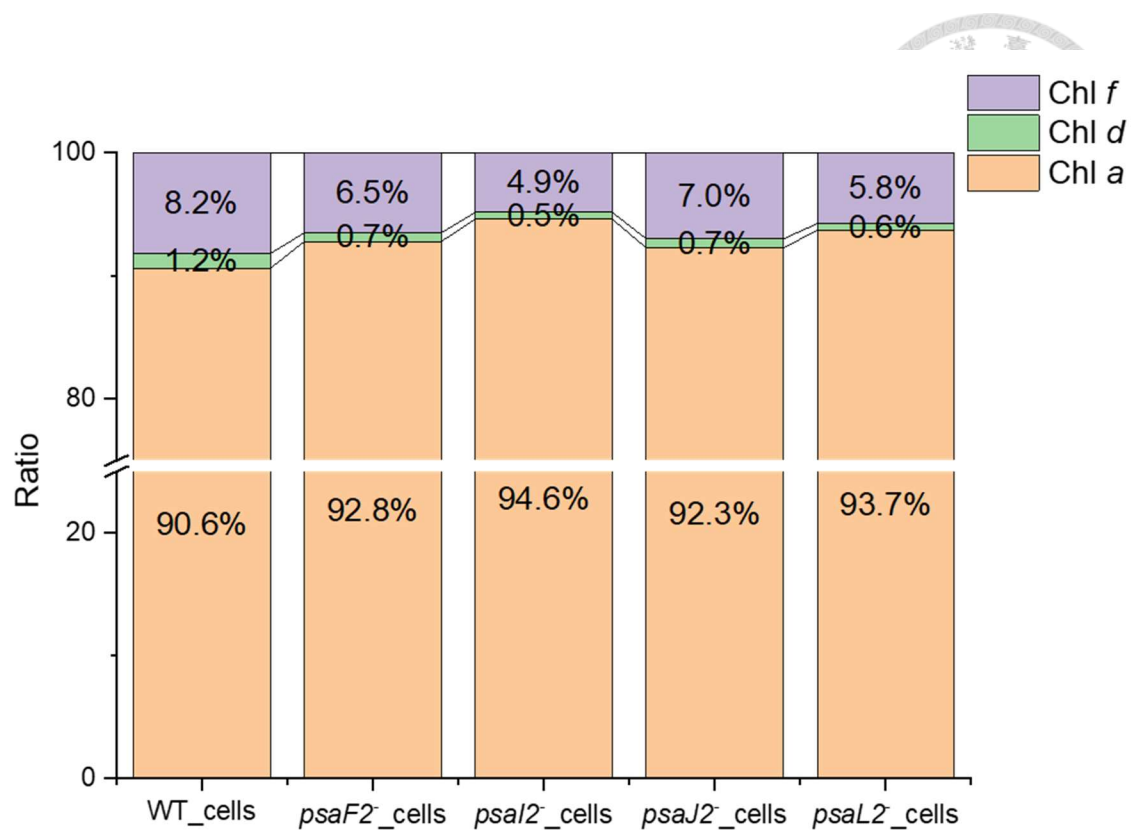


Appendix



Appendix 1 Absorbance spectra of the mutants and WT cells grown under FRL

Absorbance spectra of the FRL Syn7335-WT and the mutants' (*psaF2*⁻, *psaI2*⁻, *psaJ2*⁻, and *psaL2*⁻) cells normalized to 0-1 and then normalized to the pick point of 679nm.



Appendix 2 The chlorophyll ratio of Chl *a*: Chl *d*: Chl *f* in the 100 % stacked bar chart of FRL Syn7335-WT, *psaF2*⁻, *psaI2*⁻, *psaJ2*⁻, and *psaL2*⁻ cells

The cells of the mutants (*psaF2*⁻, *psaI2*⁻, *psaJ2*⁻, and *psaL2*⁻) were grown under FRL with $\sim 100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (centered at 730 nm). In contrast, the FRL Syn7335-WT cells were grown under FRL with $\sim 50 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (centered at 730 nm) and had a higher FRL to WL ratio, and were acclimated to FRL more completely.