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Mutagenesis of Ser^{24} of cytochrome b_{559} α subunit affects PSII activities in *Chlamydomonas reinhardtii*

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In order to study the functions of cytochrome b_{559} (Cyt b_{559}) in photosystem two (PSII) activity, mutant S24F of *Chlamydomonas reinhardtii* was constructed using site directed mutagenesis, in which Serine^{24} (Ser^{24}) locating downstream of Histidine^{23} (His^{23}) in α subunit of Cyt b_{559} was replaced by Phenylalanine (Phe). Physiological and biochemical analysis showed that mutant S24F could be grown photoautotrophically or photoheterotrophically. However, their growth rate was slower either on HSM or TAP medium than that of the control; Analysis of PSII activity revealed that its oxygen evolution was about 71% of wild type (WT); The Photochemical efficiency of PSII (F_v/F_m) of S24F was reduced 0.23 compared with WT; S24F was more sensitive to strong light irradiance than the wild type; Furthermore, SDS-PAGE and Western-blotting analysis indicated that the expression levels of α subunit of Cyt b_{559}, LHCII and PsbO of S24F were a little less than those of the wild type. Overall, these data suggests that Ser^{24} plays a significant role in making Cyt b_{559} structure maintain PSII complex activity of oxygen evolution although it is not directly bound to heme group.

Chlamydomonas reinhardtii, chloroplast transformation, cytochrome b_{559}, site-directed mutagenesis, photosystem two

Photosystem two (PSII) is a pigment-protein complex in the thylakoid membranes of higher plants, algae and cyanobacteria. It catalyzes the photosynthetic oxidation of water to dioxygen and the reduction of plastoquinone[1] and it also plays a great role in the photosynthetic electron transport chain[2,3]. The core of PSII is the reaction center complex (RC) composed of the polypeptides D1, D2, PsbI, and a transmembrane *b*-type cytochrome termed cytochrome b_{559} (Cyt b_{559})[4].

Cyt b_{559} is a hemachrome protein and a ubiquitous component of the most minimal PSII reaction center complexes (PSII-RC). It is composed of a heme group and two small polypeptides, α (9.3 kD) and β (4.4 kD) subunits with a stoichiometry of 1∶1. Both polypeptides contain a hydrophobic domain that is assumed to have a transmembrane α-helical structure. α (83 aa) and β (49 aa) subunits were encoded by *psbE* gene and *psbF* gene respectively[5]. The hydrophilic, genetic and spectroscopic analysis showed that the heme group of Cyt b_{559} is ligated by a single Histidine (His) residue in each subunits and the orientation of heme group is shown to be perpendicular to the membrane plane. Cyt b_{559} is located in close proximity to D1/D2 in PSII[6]. And the X-ray crystallography studies of *Synechococcus elongates* PSII crystal further confirmed that Cyt b_{559} heme-iron is about 27.0 Å apart from ChlzD2, and about 8 Å apart from the stromal side[7].

The research of Cyt b_{559} functions in PSII has achieved mighty advances during the past two decades. The studies of PSII-RC isolated from higher plants indicated that Cyt b_{559} is essential for the assembly of functional PSII complexes. Suggested functions include:

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(1) a role in the photoprotection of PSII, which Cyt b_{559} copes with excess excited energies by mediating cyclic electron transfer around the PSII-RC and prevents photoinhibition damage in the donor and acceptor sides[8,9]; (2) a necessary element in the assembly of stable PSII[10,11]; (3) participating in the water oxidation and reduction[12]. Recently, it has also been suggested that Cyt b_{559} may function as an electron donor to plastoquinones[13]. However, the analysis of Cyt b_{559} with regard to PSII functions, particularly *in vivo*, has been hindered by the complexities of Cyt b_{559} and a lack of appropriate Cyt b_{559} mutants that still accumulate active PSII[11].

Previous studies mainly focused on the functions of two His residues with ligand to the heme in Cyt b_{559}[10,11,14,15] and there are few reports about the functions of other amino acids in Cyt b_{559} α and β subunits. Till now, only one paper reported the changes of photosynthesis characteristics and PSII activities in the tobacco mutant, in which Phenylalanine (Phe) close to His in β Cyt b_{559} subunit was replaced by Serine (Ser)[13]. Here we described the mutant S24F of *Chlamydomonas reinhardtii*, in which Ser^{24}, next to His^{23} in α subunit of Cyt b_{559}, was replaced by Phe using site-directed mutagenesis technique. Our results suggested that Ser^{24} close to heme group plays a great role in maintaining the normal structure of Cyt b_{559} and the assembly of functional PSII complexes.

1 Materials and method

1.1 *C. reinhadtii* strains and growth conditions

C. reinhardtii wild type (WT) strain CC-125 mt^+ was obtained from Prof. Harris EH (the *Chlamydomonas* Genetic Center, Department of Botany, Duke University, Durham, NC). The WT strains of *C. reinhardtii* were grown in Tris-acetate-phosphate (TAP) medium or high salt minimal (HSM) medium at 20—25℃ under a light intensity of 50—100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 8 h/d[16].

1.2 Site-directed mutagenesis

(i) Plasmids. Plasmid p78 and plasmid p423 were provided kindly by Prof. Harris. Plasmid p78 contains the 15.1-kb *Pst* I-4 fragments, which included *psbE* gene upstream and downstream flanking sequences. Plasmid p423 contained a 1.9-kb *aadA* cassette which can supply spectinomycin resistance, and plasmid pE3 was constructed from plasmid p78 and p423 with *psbE* and *aadA* cassette in it[17].

(ii) Site-directed mutagenesis. Site-directed mutagenesis *in vitro* of the *psbE* was performed as described by Landt et al.[18], as shown in Figure 1. The sequences of primer P1 and P2 were 5′-TTACGATGTGCCAGAAA-3′ and 5′-TTTAATTTGAACTTGAGCTACCGCTTC-TTTACGAC respectively-3′. The sequence of primer MS24F was 5′-AAGCAGGTACCGTAAT*AAA*GTGA-ATAACCC-3′ in which Ser codon was substituted by the condon for Phe (in italics) and incorporated a novel *Kpn* I site (underlined). PCR were performed in an MJ PTC-200 Peltier thermal cycler (Bio-Rad Laboratories, Inc., Waltham, MA) using *Pfu* DNA polymerase (Bio Basic Inc. Shanghai, China). The final PCR fragment (1.6 kb) was digested with *Sac* I and *Acc* I, and one 0.8-kb fragment was purified and ligated into the *Sac* I/*Acc* I-restriction site of pE3, yielding the final vector, with the name of pS24F, used for transformation of *C. reinhardtii*. The inserts were sequenced by Bio Basic Inc to confirm the mutations.

1.3 Chloroplast transformation and selection of *C. reinhadtii* transformants

Plasmid pS24F was introduced into chloroplasts of *C. reinhardtii* using the biolistic method described by Boynton and Gillham[19] with BioRad Biolistic PDS-1000/He microparticle transformation system (Bio-Rad Laboratories, Inc., Hercules, CA). Plasmid pE3 was not transformed into chloroplasts as a positive control because there were no obvious differences between the control and WT[11]. Transformants were selected and re-streaked four times on selection plates (TAP+spectinomycin 100 μg/mL) before homoplasmicity identification.

1.4 Homoplasmicity identification of transformant chloroplast

Total chloroplast genomic DNA of WT and transformants was isolated using the procedures described by Lee et al.[20]. To identify mutant alleles, the *pbsE* in the transformants was amplified using primers P3 (5′-ATAGATGGTTTGAAAAGG-3′) and P4 (5′-TTG-TTTCAATGGGGCATTTA-3′) with genomic DNA as templates. The PCR fragments were separated on a 1.0% agarose gel. PCR-Southern-blot hybridization was carried out according to the manual of DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics Gmbh, Germany).

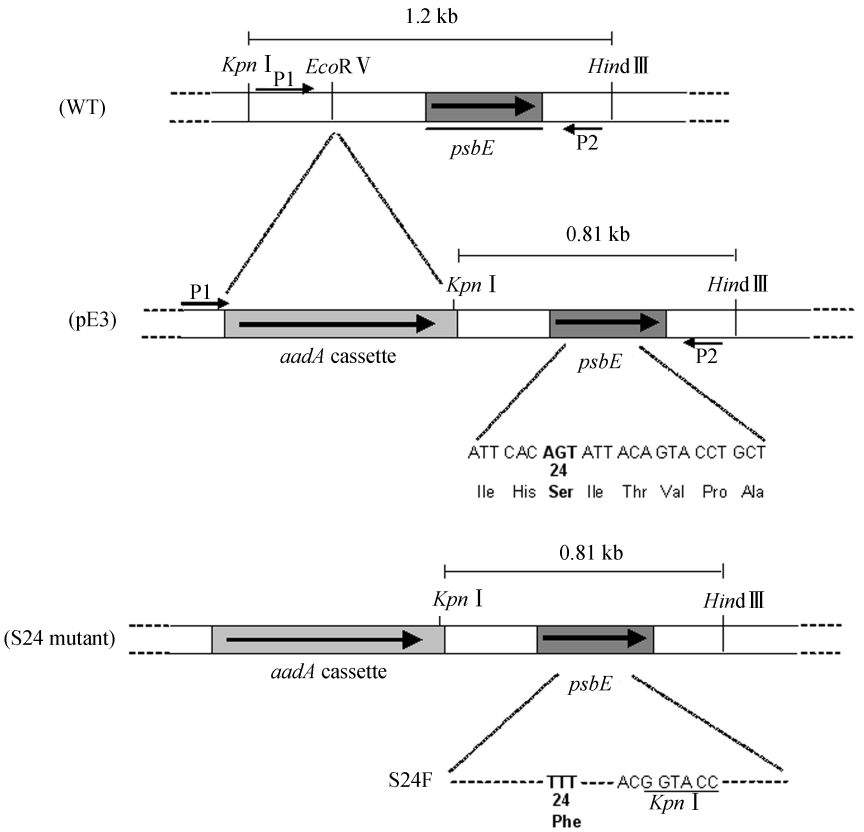

Figure 1 Construction of transformed plasmid pS24F. The directions of the *aadA* cassette and *psbE* were shown. Associated site-directed mutations of *psbE* were indicated in *boldface type*.

1.5 Growth rate and light absorbance measurements

The measurements of *C. reinhadtii* growth rate were performed as described by Fan et al.[21]. An ultraviolet-visible spectrophotometer (model MPS-2550, Shimadzu, Tokyo, Japan) was used to measure the A_{750} of *C. reinhadtii* cells.

1.6 Assay of oxygen evolution

Oxygen evolution for PSII activity of whole *C. reinhadtii* cells was measured at 25℃ and saturating light intensity (1000 μmol·m^{-2}·s^{-1}) with a Clark-type O_2 electrode (Hansatech, King's Lynn, UK), using the methods described by Leong[22].

1.7 Measurements of fluorescence induction kinetics parameters

Fluorescence induction kinetics parameters in whole cells of WT and transformant strains were measured using the methods of Genty et al.[23] with some modifications. Cells grew in TAP medium till the A_{750} reached 0.6—0.8. The samples were dark-adapted for 5 min prior to the measurements. At room temperature, the minimum chlorophyll fluorescence (F_0) was measured under the dim light (about 30 μmol·m^{-2}·s^{-1}), and maximum chlorophyll fluorescence (F_m) under a saturating pulse light (1000 μmol·m^{-2}·s^{-1}) for about 0.8 s, using a pulse-amplitude-modulated fluorometer (PAM 101, Walz, Effeltrich, Germany). Finally, $F_v/F_m[(F_m–F_0)/F_0]$, the Photochemical efficiency of PSII, was calculated. Each sample was repeated 5—10 times.

1.8 Photoinhibition measurements

The mutated and WT cells of *C. reinhardtii* were grown in TAP liquid medium to A_{750} of 0.6—0.8. The cultures were then subjected to moderate illumination of 200 μmol·m^{-2}·s^{-1} or high illumination of 1000 μmol·m^{-2}·s^{-1} at 25℃. 3 mL samples were taken at 10 min intervals over a period of 30 min and their oxygen evolution rates at saturating light intensity (1000 μmol·m^{-2}·s^{-1}) were analyzed.

1.9 SDS-PAGE, Western blotting and absorbance spectrum of thylakoid membranes

Thylakoid membranes were prepared using the methods of Ruffle et al.[24]. After SDS-PAGE, half of gel was stained with Coomassie Brilliant Blue R-250, and an-

other half was used for Western blotting analysis. Both SDS-PAGE and the immunodetection of proteins were performed using the method described by Sambrook et al.[25], with antibodies specific for Cyt b_{559} α subunit (a gift from Prof. Barber J, Department of Biochemistry, Imperial College of Science, Technology, and Medicine, London, United Kingdom), LHCII and PsbO (provided by Prof. Zhang LiXin, the Institute of Botany, Chinese Academy of Sciences). The relative amount of each band in Western blotting was calculated using the software Labworks (Ultra-Violet Products Ltd, America) with 3 repeats. The room temperature absorbance spectrum of 10 μg Chl/mL of thylakoid membranes was carried out in the dark by UVKON-943 double beam spectrophotometer, with the scan rate of 100 nm/min and 0.5 nm resolution.

2 Results

2.1 Construction of chloroplast homologous recombinant plasmid pS24F

Plasmid pS24F was constructed as described below: The PCR fragment containing the mutated sites was digested with restriction endonuclease *Sac* I and *Acc* I to produce a 0.8-kb fragment. After purified, the 0.8-kb fragment was ligated into the *Sac* I/*Acc* I-restriction site of pE3, yielding the final vector pS24F containing *psbE* gene with the engineered mutations and *aadA* cassette in it. The mutations in plasmid pS24F were confirmed by DNA sequencing (Bio Basic Inc., Shanghai, China).

2.2 Selection and homoplasmicity identification of *C. reinhadtii* transformants

C. reinhadtii transformants (named S24F) were selected and re-streaked four times on TAP plates containing 100 μg/mL spectinomycin before being further analyzed. To identify mutant alleles, the *pbsE* gene in the chloroplast genomic DNA of WT and S24F was amplified using primers P3 and P4, which were located at upstream and downstream of *pbsE* gene respectively. The PCR products from WT were about 0.75 kb (lane 2, Figure 2(a)) while those from the S24F mutants, shown in lane 3 (Figure 2(a)), were 2.6 kb in length (no 0.75 kb fragment appeared). The results showed that the *aadA* cassette (about 1.9 kb) was incorporated into the chloroplast genome of S24F via homologous recombination and it also indicated that chloroplasts in S24F were homoplasmic. PCR products of WT and S24F were digested with *Kpn* I respectively and the results showed that only the PCR products of S24F can be digested by *Kpn* I and two anticipated fragments of about 0.2 and 0.4 kb appeared (lanes 4 and 5, Figure 2(a)). Furthermore, in order to confirm the specificity of PCR products, they were transferred onto nylon membranes and hybridized by a *psbE*-specific probe. The results (lanes 2 and 3, Figure 2(b)) indicated that PCR products were the fragments of *psbE* gene. All the results showed that the chloroplast genome in S24F contained the *psbE* gene with the engineered mutations and the mutants appear to be highly homoplasmic without wild type chloroplasts.

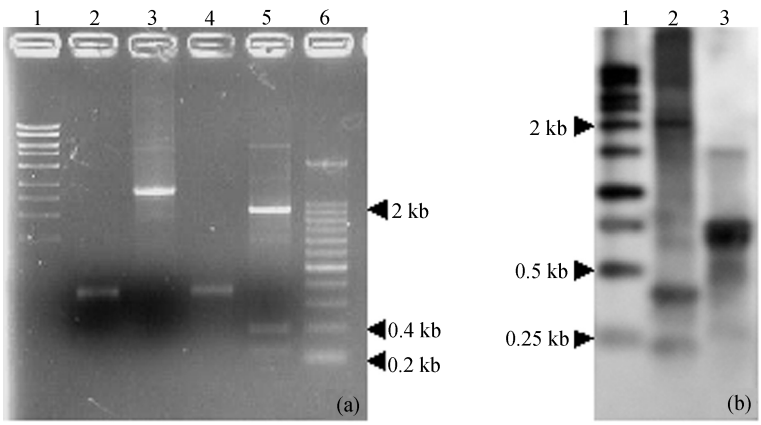

Figure 2 PCR analysis with primers P3 and P4 and PCR-Southern blotting of S24F and WT. (a) Electrophoresis of PCR products amplified from WT and S24F. Lane 1, 0393 ladder; lanes 2 and 3, PCR products of WT and S24F respectively; lanes 4 and 5, results of PCR products from WT and S24F digested by *Kpn* I respectively; lane 6, 200 bp DNA ladder. (b) PCR-Southern blotting analysis. Lane 1, 1 kb DNA ladder; lanes 2 and 3, PCR products of S24F and WT hybridized with *psbE*-specific probe after digested by *Kpn* I respectively.

2.3 Growth properties and photosynthetic characteristics of S24F

Mutant strain S24F can be grown photoautotrophically and photoheterotrophically, but both the growth rates were slower than those of WT (Table 1). The time period to reach stationary phase for mutant strains in HSM medium or in TAP medium were 4 or 8 d more than those of WT respectively. The F_v/F_m ratio (Photochemical efficiency of PSII) of mutant S24F was 0.23 lower than that of WT, and PSII oxygen evolution rates for the mutants were 71% of the wild type.

2.4 S24F is more susceptible to photoinactivation

When S24F and WT cells were exposed to moderate intensity white light (200 μmol·m^{-2}·s^{-1}), the PSII activities of S24F during the time course of the experiment were dramatically decreased, and the PSII activities of the mutants remained only 68% of the total activities after 30 min exposure, while those of WT just showed a little bit of decrease. Under high intensity light condition, PSII activities of the mutants were undetected after 10 min exposition, while those of WT even stayed at 35% after 30 min exposition (Table 2). Those results showed that mutant strain S24F was more susceptible to photoinactivation than WT.

2.5 Analysis of S24F thylakoid membranes

Thylakoid membranes of the mutant S24F gave the room temperature absorbance spectra with three peaks at 440, 460 and 676 nm similar to those of WT thylakoid membranes (Figure 3(b) and (c)). SDS-PAGE (Figure 3(a)) and Western blotting analysis (Figure 4) showed that the profiles of thylakoid membrane proteins in the mutant strains were expressed similarly to those in WT cells. However, the expression levels of Cyt b_{559} α subunit, PsbO and LHCII were reduced in thylakoid membranes isolated from the *psbE* mutant cells compared with WT. And the expression levels of Cyt b_{559} α subunit and PsbO in S24F were only 80% of the wild type.

3 Discussion

Chlamydomonas is a unicellular green alga with a short and simple life cycle. It can be grown photoautotrophically and photoheterotrophically and it is easy to perform the chloroplast and nuclear transformation with high efficiencies. So it has become a model system for molecular mechanism studies of eukaryotic development, especially in the areas where yeast cannot be used, such as in photosynthesis and biogenesis of the chloroplast studies[16]. Furthermore, in *cyanobacteria* and higher plants, the *psbEFLJ* forms one operon, while in *C. reinhardtii*, these genes are split into two separate transcription units which locate at different regions of chloroplast genome and they are separately transcribed in the opposite direction[26]. This makes it possible to disrupt or mutate the *psbE* gene without affecting transcription of *psbF* or other genes. So in this research, *C. reinhardtii* was used to construct the mutants of Cyt b_{559}.

Cyt b_{559} is a ubiquitous heme protein in the most minimal PSII complexes. And it is essential for assembly of stable PSII. In order to study the structures and functions of Cyt b_{559}, many mutants were constructed, such as mutants of *Syncchocystis* 6803 in which *psbE* or *psbF* gene was replaced by *Km*^{r} gene[14,27]; mutant of *C. reinhardtii* in which the *aadA* cassette was inserted into *psbE*[10]; mutant of His^{22}/Leucine mutation in *Synechocystis* 6803[15] and mutants H23M, H23Q and H23Y of Cyt b_{559} α subunit constructed with *C. reinhardtii*[11]. But those mutations resulted in expression level reductions of some components in PSII complexes and partial or complete loss of PSII activities. The exact functions

Table 1 Growth properties and photosynthetic characteristics of WT and mutant strain cells

Strain	Days reaching to stationary phase		F_0	F_m	F_v/F_m	Oxygen evolution (μmol O_2·mg Chl^{-1}·h^{-1})
	HSM	TAP				
CC-125	4	8	0.257±0.048	0.908±0.270	0.706±0.045	150±8.3
S24F	8	16	0.264±0.068	0.514±0.125	0.475±0.071	106±10.2

Table 2 Effects of moderate and high light intensity on oxygen evolution of WT and the mutant cells

Oxygen evolution (μmol O_2·mg Chl^{-1}·h^{-1})		Time of exposition (min)			
		0	10	20	30
Moderate light intensity (200 μmol·m^{-2}·s^{-1})	CC-125	150±8.3	147±7.7	143±12.3	128±12.8
	S24F	106±10.2	77±8.9	68±11.2	53±11.3
High light intensity (1000 μmol·m^{-2}·s^{-1})	CC-125	150±8.3	137±4.26	123±7.56	99±6.63
	S24F	106±10.2	0	0	0

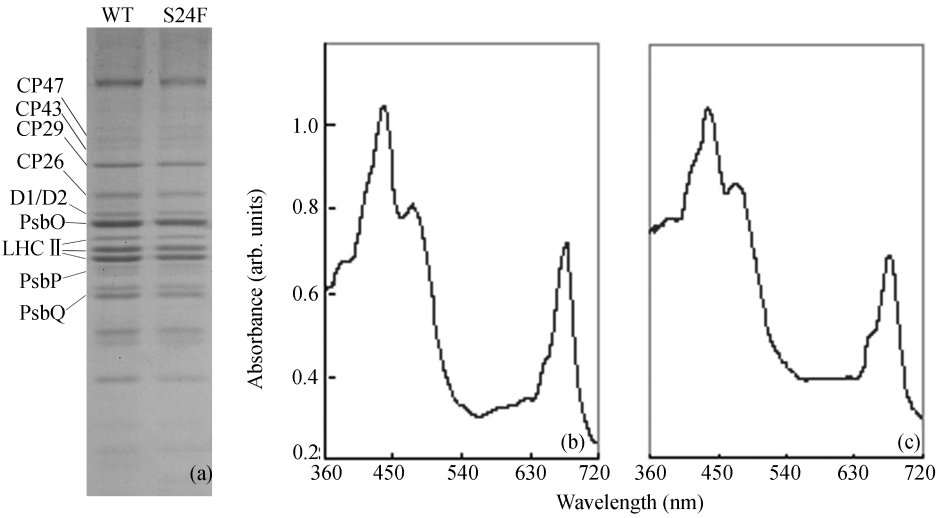

Figure 3 Thylakoid membranes room temperature absorbance spectra and SDS-PAGE of mutant S24F and WT. (a) SDS-PAGE of WT and the mutant S24F; (b) and (c), thylakoid membranes room temperature absorbance spectra of WT and S24F.

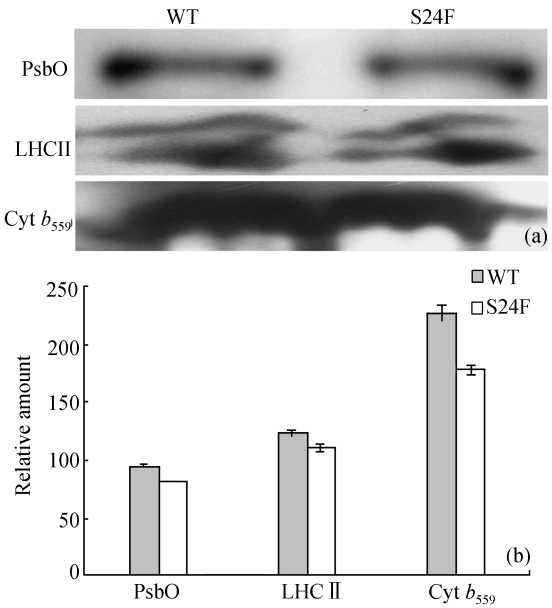

Figure 4 Immunoblots of thylakoid membranes isolated from WT and S24F with antibodies specific for α-Cyt b_{559}, PsbO and LHCII polypeptide (a) and relative amount of each band for WT and S24F in Western blotting (b). All samples contain 3 μg of chlorophyll.

of Cyt b_{559} in PSII are still unclear for lack of appropriate Cyt b_{559} mutants with highly active PSII complexes[11].

However, the previous studies on mutants of Cyt b_{559} focused particularly on the mutations of heme-ligating His residue, but rarely on other amino acid mutations in α and β subunits of Cyt b_{559}. Only Bondarava et al.[13] indicated that the PSII activities of the *tobacco* mutant in which Phe^{26}/Ser mutation occurred in Cyt b_{559} β subunit remain 40% of PSII activities of the wild type. Here, Cyt b_{559} mutant S24F was constructed using site-directed mutagenesis technique, in which Ser^{24} next to His^{23} of Cyt b_{559} α subunit was replaced by Phe residue. The data of growth rate and photosynthetic characteristic analysis (Table 1) showed that S24F can be grown photoautotrophically or photoheterotrophically but only with a 50% growth rate of WT. The Photochemical efficiency of PSII (F_v/F_m) and oxygen evolution of the mutants were lower than those of WT, indicating that the photosynthesis of mutant cells was influenced by the mutagenesis. The sensitivity measurement to strong light (Table 2) suggested that S24F was more sensitive to strong light than WT. Though the study of the growth rate, photosynthetic characteristics and sensitivity to strong light in S24F showed similar results as His^{23} mutants[11], the PSII activities of S24F and the *tobacco* mutant (Phe^{26}/Ser) are higher than those of other Cyt b_{559} mutants, suggesting the weaker are the influences on PSII activities of Ser^{24} mutation than His^{23} mutations. The probable reason was that His^{23} is directly bound to prosthetic group heme.

Cyt b_{559} null mutants and the mutants derived from His residue mutations in α or β subunits could seriously affect the expression levels of some components in PSII complexes, such as D1, D2, CP47, psbO, α and β subunits of Cyt b_{559}, and result in no assembly of PSII, or partial or complete loss of PSII activity in these mutants. It was found that D_1, D_2, CP43 and CP47 were severely diminished when His^{22} in Cyt b_{559} α and β subunits of *Syncchocystis* were replaced by Leu separately or simultaneously, and Cyt b_{559} α subunit in the mutants (one-site mutation) was 1.5 kD smaller than that of WT.

It was also reported that the expression levels of D1, D2, Cyt b_{559} α and β subunits remain 10%—25% of WT after His[23] of Cyt b_{559} α subunit in *C. reinhardtii* was substituted with Methionie and Tyrosine residues[10,11,14,15,27]. Furthermore, changes of Phe[26] in Cyt b_{559} β subunit resulted in the low expression levels of CP47, α and β subunits of Cyt b_{559} proteins and caused the assembly of PSII in thylakoid membrane reduced[13]. The results of SDS-PAGE and Western blotting analysis (Figures 3 and 4) showed that the expression level of LHC II was reduced and the amount of PsbO and α subunits of Cyt b_{559} in S24F were about 80% of WT, close to the results from other mutants. But the results also showed that Ser[24] mutations in α subunit and Phe[26] mutations in β subunit of Cyt b_{559} had smaller influences on the expression of some components in PSII complexes than those caused by His changes.

In one word, the results presented here demonstrated that Ser[24] is necessary for keeping the functions of Cyt b_{559} and it also plays a significant role in maintaining PSII activities although Ser[24] is not directly bound to prosthetic group heme. Moreover, it will be helpful to know much more structures and functions of Cyt b_{559} in PSII if the mutant S24F with higher PSII activities is further studied in the area of electron-transfer reaction and dynamics characters during the course of photoxidization and photoreduction.

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