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Supramolecular architecture of photosynthetic membrane in red algae in response to nitrogen starvation

Long-Sheng Zhao [a], Hai-Nan Su [a,*], Kang Li [a], Bin-Bin Xie [a], Lu-Ning Liu [a,1], Xi-Ying Zhang [a], Xiu-Lan Chen [a], Feng Huang [a], Bai-Cheng Zhou [a], Yu-Zhong Zhang [a,b]

[a] State Key Laboratory of Microbial Technology, Marine Biotechnology Research Center, Institute of Marine Science and Technology, Shandong University, Jinan 250100, China
[b] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

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ABSTRACT

The availability of nitrogen is one of the most important determinants that can limit the growth of photosynthetic organisms including plants and algae; however, direct observations on the supramolecular architecture of photosynthetic membranes in response to nitrogen stress are still lacking. Red algae are an important evolutionary group of algae which contain phycobilisomes (PBSs) on their thylakoid membranes, as do cyanobacteria. PBSs function not only as light-harvesting antennae but also as nitrogen storage. In this report, alterations of the supramolecular architecture of thylakoid membranes from red alga *Porphyridium cruentum* during nitrogen starvation were characterized. The morphology of the intact thylakoid membrane was observed to be round vesicles. Thylakoid membranes were reduced in content and PBSs were degraded during nitrogen starvation. The size and density of PBSs were both found to be reduced. PBS size decreased by less than one-half after 20 days of nitrogen starvation, but their hemispherical morphology was retained. The density of PBSs on thylakoid membranes was more seriously affected as time proceeded. Upon re-addition of nitrogen led to increasing of PBSs on thylakoid membranes. This work reports the first direct observation on alterations in the supramolecular architecture of thylakoid membranes from a photosynthetic organism in response to nitrogen stress.

1. Introduction

The phycobilisome (PBS) is the main light harvesting complex in cyanobacteria and red algae [1,2], and it is among the largest light harvesting antennae in all photosynthetic organisms with an estimated molecular weight of 4.5–23 × 10^6 Da [3,4]. PBSs are attached to the stromal side of thylakoid membranes in a highly aggregated form and differ from the light harvesting complexes in green plants which are integrated in thylakoid membranes [5,6]. PBSs absorb and transfer solar energy with high efficiency to the photosystems that are located within thylakoid membrane [7–9]. The content of PBSs in cells is known to vary depending upon the growth conditions, and under optimal growth conditions, PBSs could account for half of all of the total soluble protein in the cells [10,11].

PBSs are composed of chromophore-linked phycobiliproteins and colorless hydrophobic linker polypeptides. Phycobiliproteins can be categorized into four main groups based on their spectra properties: allophycocyanin (APC), phycocyanin (PC), phycoerythrin (PE) and phycoerythrocyanin (PEC) [12]. Along with linker polypeptides, phycobiliproteins assemble into high molecular mass complexes, the PBSs [13]. Based on electron microscopic observations, PBSs can be sorted into three topological types: hemidiscoidal, hemispherical and bundle-shaped [14]. Hemidiscoidal PBSs are found in both cyanobacteria and red algae, and hemispherical PBS are in red algae [15]. Bundle-shaped PBSs are found in only one species of cyanobacteria, *Gloeobacter violaceus* [16].

Transmission electron microscopy (TEM) is one of the most important tools for studying the ultrastructure of thylakoid membranes and their associated supramolecular complexes in photosynthetic organisms. TEM provides abundant direct observations and promotes photosynthesis research. In recent years, another important tool, atomic force microscopy (AFM), has proven to be a unique and powerful method for studying the supramolecular architecture of native photosynthetic membranes under near-physiological conditions [17]. Although photosynthetic membranes from purple bacteria [18,19] and higher plants [20–24] have been studied in detail with AFM, little research has been performed on the photosynthetic membranes of cyanobacteria or algae with AFM. Our previous study has reported the first AFM observation of the native supramolecular architecture of the photosynthetic membranes from the red alga *Porphyridium cruentum*

Abbreviations: AFM, atomic force microscopy; APC, allophycocyanin; ASW, artificial seawater; —NASW, artificial seawater medium lacking nitrate; PBS, phycobilisome; PC, phycocyanin; PE, phycoerythrin; PEC, phycoerythrocyanin; TEM, transmission electron microscopy.

* Corresponding author.
E-mail address: suhn@sdu.edu.cn (H.-N. Su).
[1] Present address: Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, United Kingdom.

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[25]. High resolution three-dimensional tomographs of thylakoid membranes showed that hemispherical PBSs are crowded on membranes and have diverse distribution patterns under different light intensities [25]. Such crowd arrangement could act as a major impediment to the lateral diffusion of PBSs on red algal thylakoid membrane [26].

In a natural environment, photosynthetic organisms, including cyanobacteria and red algae, have developed a variety of adaptive responses to cope with stressful conditions, including nutrient limitation or even deprivation, and survive under changing environmental conditions [1]. A series of ordered and complicated physiological reactions occur in non-diazotrophic cyanobacteria and red algae to allow survival under conditions of nitrogen deficiency [27–29]. During nitrogen starvation, the PBS is degraded to provide a nitrogen source for protein synthesis and other cellular functions. Moreover, degradation of PBSs reduces the absorption capacity and thus to prevent photodamage caused by overexcitation [30].

Although nitrogen availability is one of the most important determinants that limits the growth of photosynthetic organisms, direct observations on the dynamics of supramolecular architecture of photosynthetic membranes from plants or algae in response to nitrogen stress are still lacking. In this study, we investigated isolated thylakoid membranes from the red alga *P. cruentum* using AFM and other techniques to reveal the changes in the supramolecular architectures of the thylakoid membranes of *P. cruentum* during nitrogen starvation. The AFM results are conducive to a better understanding of the alterations of thylakoid membranes and the mechanism of PBS degradation under nitrogen stress. This work reports the first direct observation on alterations in the supramolecular architecture of thylakoid membranes from a photosynthetic organism in response to nitrogen stress.

2. Experimental procedures

2.1. Cell growth

P. cruentum was grown in an artificial seawater (ASW) medium [31] in flasks at 25 °C under continuous illumination (50 μmol quanta m^{-2} s^{-1}). Cells that grew to late logarithmic phase in nitrogen-rich ASW medium were concentrated by centrifugation (10 min at 5000*g* at room temperature). The pellet was washed three times in ASW medium that lacked potassium nitrate and was then suspended in ASW medium lacking nitrate (−NASW) at a ratio of 0.1 g (wet weight) cells: 1 ml medium. Two milliliters of the suspension was added to 200 ml of nitrogen-rich ASW medium in the control treatment, and 2 ml of the suspension was added to 200 ml of −NASW medium in the nitrogen-starvation treatment. Cells that were collected from control cultures in the late logarithmic phase of growth, and nitrogen-starved cultures at 0, 5, 10, 15 and 20 days were used in following experiments. For nitrogen recovery cultivation, potassium nitrate (0.1%, w/v) was added to *P. cruentum* cultures after 20 days nitrogen starvation. Cells grown to 6 days were collected for the following experiments.

2.2. PBS isolation

The preparation of PBSs followed a previous procedure with slight modifications [32]. The entire procedure was performed at room temperature in 0.75 M potassium phosphate (pH 7.0) without any special instructions. Cells were harvested by centrifugation at 5000*g* for 10 min and were then rinsed twice in 0.5 M potassium phosphate (pH 7.0). The pellets were suspended in buffer at a ratio of 0.1 g (wet weight) cells: 1 ml buffer and then disrupted in a high-pressure homogenizer at 10,000 p.s.i. Triton X-100 was added to the cell homogenate to a final concentration of 2% (v/v). The mixture was allowed to incubate for 30 min with stirring using vertical rotators, and then centrifuged at 25,000*g* for 30 min to remove large fragments and cell debris. The dark green superstratum was removed. The clarified middle samples were then layered onto a three-step gradient of sucrose (0.5 M, 1.0 M and 2 M) in buffer. The gradients were centrifuged for 1.5 h at 147,000*g*. PBSs were collected from the 1.0 M layer using a syringe needle.

2.3. PBS-thylakoid membrane preparation

PBS-thylakoid membranes were prepared by following a previous procedure with slight modifications [25]. Cells were harvested, rinsed as described above, and then suspended in SPC medium (0.5 M sucrose, 0.5 M potassium phosphate, 0.3 M sodium citrate). The cells were disrupted in a high-pressure homogenizer at 4000 p.s.i. The disrupted cell mixture was layered onto a two-step gradient of sucrose (0.7 M and 1.3 M) that was prepared with 0.5 M phosphate/0.3 M citrate, pH 7.0. The PBS-thylakoid membranes were collected from the 0.7–1.3 M sucrose interface after centrifugation for 40 min at 300,000*g*. For preparing PBS-free thylakoid membranes, isolated thylakoid membranes were dialyzed against buffer (30 mM potassium phosphate, 20 mM sodium citrate) for 7 h.

2.4. Spectral analysis

Absorption spectra were recorded at room temperature using a UV/VIS-550 spectrophotometer (Jasco, Japan). Room temperature fluorescence spectra were measured using a FP-6500 fluorescence spectrofluorometer (Jasco, Japan) at an excitation wavelength of 545 nm.

2.5. AFM

Isolated PBS-thylakoid membranes from the sucrose gradient were dialyzed against 0.5 M phosphate/0.3 M citrate (pH 7.0) to remove the sucrose. Adsorption buffer, 13 μl, (10 mM Tris–HCl, pH 7.5, 150 mM KCl, 25 mM $MgCl_2$) [33] mixed with 2 μl of 10% glutaraldehyde was spread on freshly cleaved mica. Five microliters of dialyzed sample was immediately injected into the buffer drop and then incubated for 1 h in a humidor. For PBS-free thylakoid membranes, 5 μl of sample was spread on freshly cleaved mica and incubated for 1 h in a humidor, without treatment described above. Then, the drop on the mica was absorbed, and the surface was rinsed with ultrapure water to remove salt and membranes that were not firmly adsorbed to the substrate. Afterwards, the samples were air dried and examined using a Multimode Nanoscope VIII AFM (Bruker AXS, Germany) equipped with a J-scanner and an n-type silicon cantilever (XSC11/AL BS; length 210 μm; resonance frequency 80 kHz; $k = 2.7$ N/m; NanoAndMore Corp, USA). Imaging was conducted in tapping mode at ambient temperature.

Comparisons between mean values of sizes and density of PBS were determined by Student's *t*-test with $P < 0.05$.

2.6. TEM

The procedure followed that in a previous study [34]. Cells were harvested, rinsed, and fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) for 2 h, followed by post-fixation in 1% osmium tetroxide (in 0.1 M phosphate buffer, pH 6.8) for 2 h. Then, the cells were rinsed in buffer, suspended in melted 2% agar, dehydrated in a graded ethanol dehydration series and embedded in Epon. Sections were stained with uranyl acetate and lead, and were then examined with an electron microscopy.

3. Results

3.1. Spectral analyses

When grown in nitrogen rich medium, the color of a culture changes from light red to a darker color, indicating growth of *P. cruentum* cells (Fig. S1). When grown in nitrogen depleted medium, the color of the

culture changed gradually to yellow-green, indicating the degradation of the phycobiliproteins (Fig. S1).

The absorption spectra of *P. cruentum* cells and thylakoid membranes during nitrogen-starvation were measured (Fig. 1A, B). The results with *P. cruentum* showed typical absorption spectra. The absorption peak at 545 nm is attributed to PE, and the absorption peak at 620 nm to PC and APC. Compared to PC/APC, the absorption peak of PE decreased more dramatically. After 20 days of nitrogen starvation, the absorption of the phycobiliproteins decreased to a fairly low level.

The absorption spectra of isolated PBSs during nitrogen starvation were measured (Fig. S2A). Absorption peaks from PE, PC and APC were observed, indicating that all three phycobiliprotein components were retained in the PBSs during nitrogen-depleted cultivation. The absorption of PE decreased gradually. Difference in absorption spectra (Fig. S2B) indicated that the changes in PBS absorbance resembled the absorption spectra of pure PE, which suggests that the content of PE in PBSs was reduced.

Fluorescence spectra were measured on cells and PBS-thylakoid membranes (Fig. 1C, D). When PE was excited, fluorescence emission from PE, PBS and photosystems could be observed from all cells and PBS-thylakoid membranes samples during nitrogen starvation. This indicated that the energy coupling between PBS and photosystems were retained during nitrogen starvation.

3.2. Ultrastructure of cells during nitrogen starvation

Stacked thylakoid membranes filled the plastid of the control cells in a parallel arrangement (Fig. 2A, F). After nitrogen starvation, thylakoid membranes were reduced significantly (Fig. 2B–E) and lost their parallel and close configuration features (Fig. 2G, H). The parallel lamellar areas were shrunken and the space between lamellae became larger. After 20 days of nitrogen starvation the lamellae were greatly reduced and disordered (Fig. 2E, H). Dark-staining granules were located in the periphery of the thylakoid and increased significantly during nitrogen starvation. The exopolysaccharide layer of the cells became thicker following nitrogen starvation. The average distances between adjacent thylakoid membranes increased during nitrogen starvation (Table S1), indicating changes of thylakoid membrane arrangement during nitrogen starvation.

3.3. Morphology of the thylakoid membrane

Thylakoid membranes from *P. cruentum* cultured in medium with nitrate were observed using AFM. Interestingly, we found that most of the intact thylakoid membranes were flattened vesicles (Fig. 3A). On occasion, folds appeared (Fig. 3D). The PBSs were densely packed on the thylakoid membranes, and they were randomly distributed. The PBSs exhibited a hemispherical morphology (Fig. 3B, C), as was seen in our previous study [25]. A lipid layer could be observed in some gap areas between the PBSs. Some thylakoid vesicles were found to be lying close to each other (Fig. 3D–F). Section analyses indicated that they were overlap with each other instead of connected with each other. Sometimes thylakoid vesicles with openings were observed (Fig. 3H).

3.4. PBSs on thylakoid membranes during nitrogen starvation

To study the changes of PBSs on the thylakoid membranes, the PBS-thylakoid membranes from *P. cruentum* cultured under nitrogen-

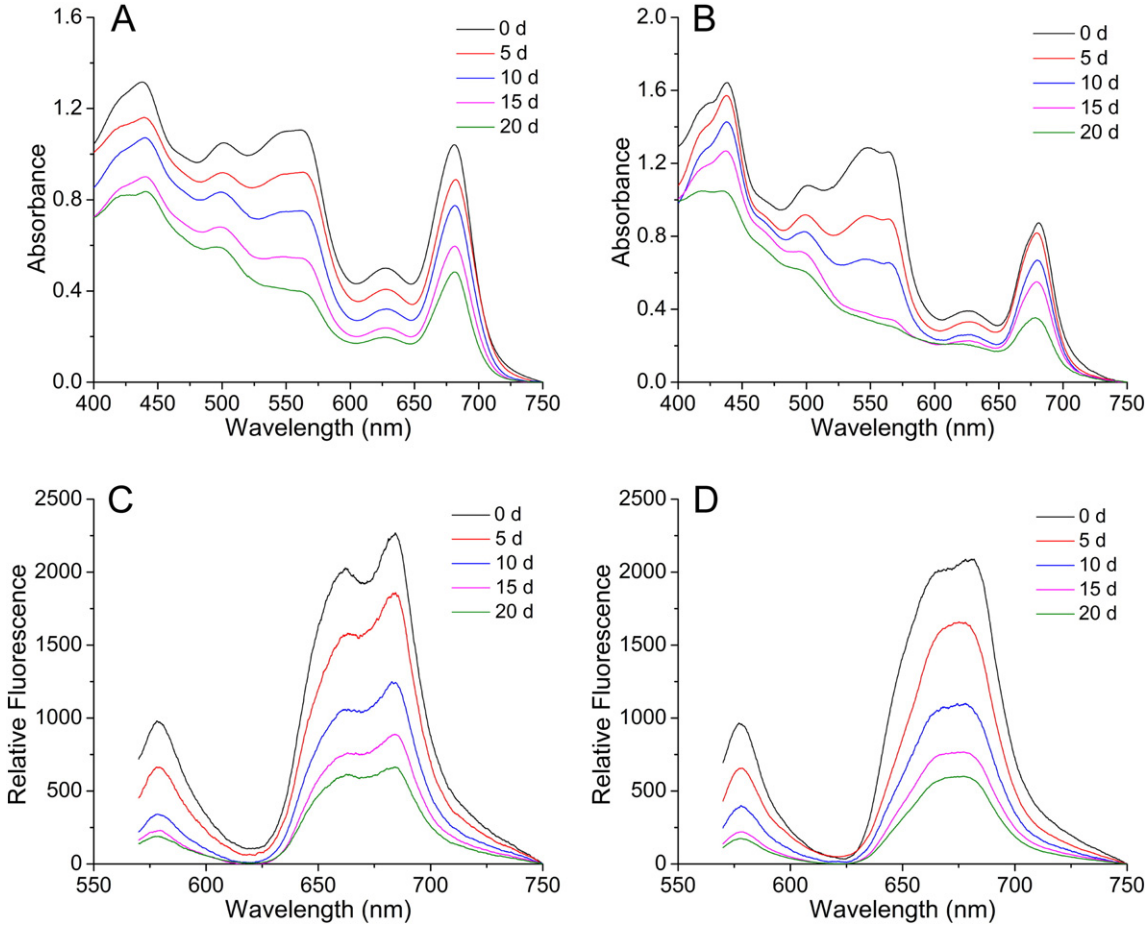

Fig. 1. Absorption spectra of cells (A), PBS-thylakoid membranes (B) from *P. cruentum* under nitrogen starvation conditions for different days (d). Fluorescence spectra of *P. cruentum* cells (C) and PBS-thylakoid membranes (D) during nitrogen starvation (excited at 545 nm).

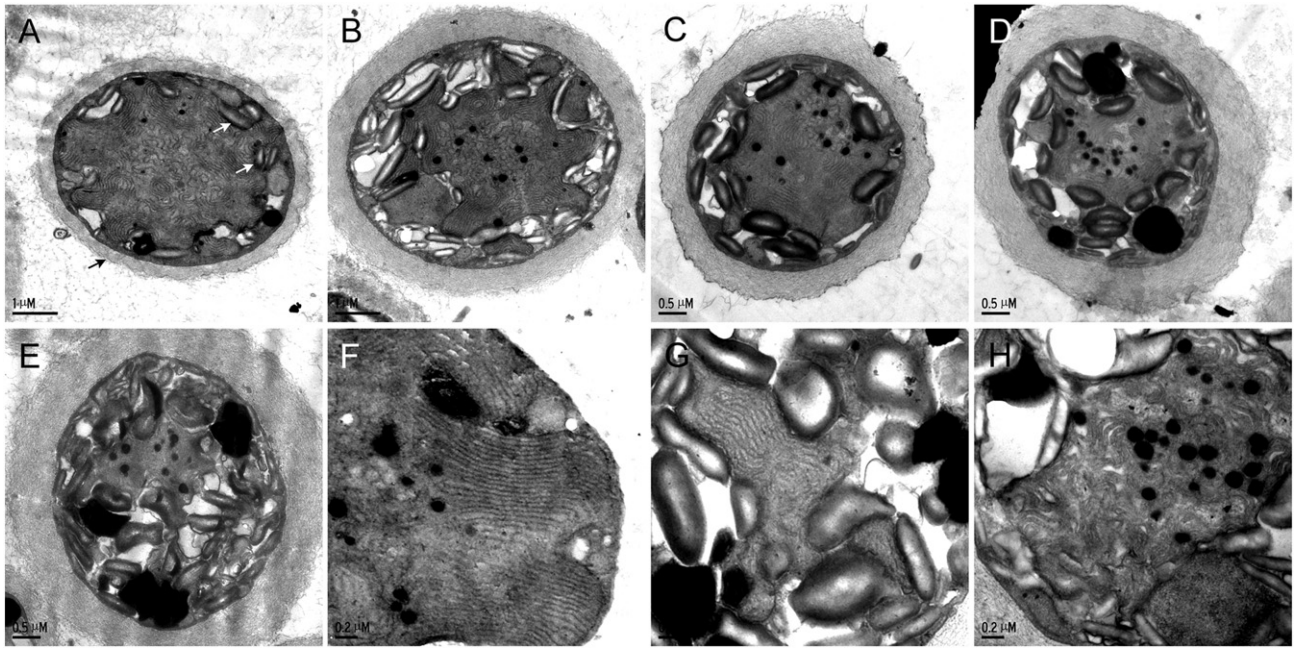

Fig. 2. TEM images of *P. cruentum* cells that were grown under nitrogen starvation for 0 (A), 5 (B), 10 (C), 15 (D) and 20 days (E). Enlarged TEM images of cells during nitrogen starvation at 0 day (F), 10 days (G), and 20 days (H). Dark-staining granules are indicated by white arrows, and the exopolysaccharide layer is indicated by a black arrow. Scale bar: A, B, 1 μm; C, D, E, 0.5 μm; F, G, H, 0.2 μm.

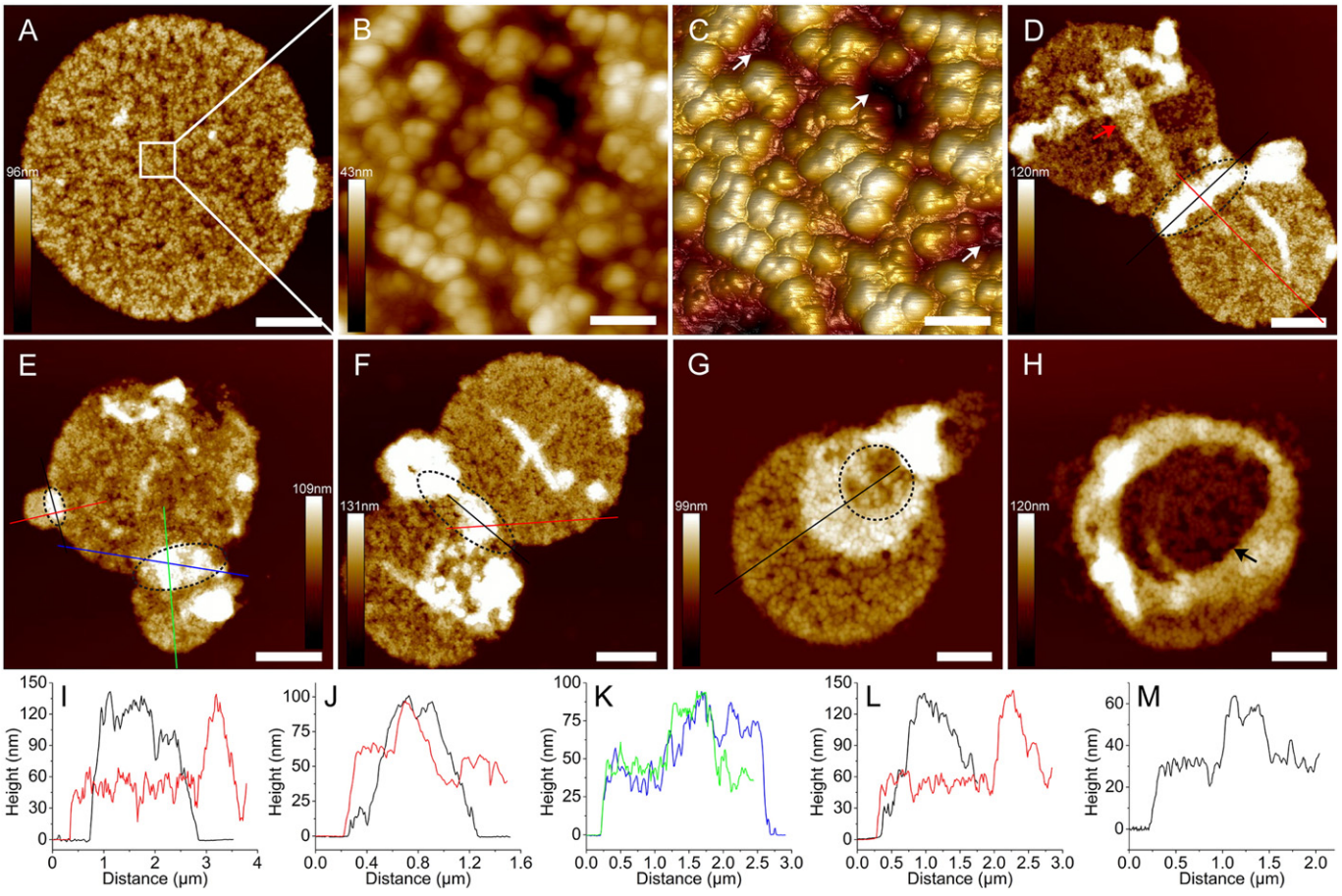

Fig. 3. AFM images of PBS-thylakoid membranes from *P. cruentum* grown under control conditions. (A), Typical round thylakoid membrane vesicles. (B), Enlarged AFM image from panel (A) with higher resolution showing the hemispherical PBS. (C), Enhanced three-dimensional AFM image of panel (B). PBS-free membrane regions are indicated by white arrows. (D–G), Thylakoid membrane vesicles that were overlapped with each other. A fold in (D) is marked with a red arrow. Junctions between two vesicles are marked with dashed circles. (H), Thylakoid membrane with an opening marked with a black arrow. Scale bar: A, D, E, F, 1 μm; B, C, 100 nm; G, H, 500 nm. (I–M) Height section analyses of thylakoid membranes at overlapped area. (I) Is height profile from (D), (J, K) from (E), (L) from (F), M from (G). Lines indicated cross section positions.

depleted conditions for 0 (control sample), 5, 10, 15 and 20 days were imaged using AFM.

During nitrogen starvation, the round morphology of the thylakoid vesicles was retained. However, statistical analysis (n = 47–88) showed that the diameters of the thylakoid vesicles increased slightly with prolonged nitrogen starvation (Table S2).

The PBS density on thylakoid membranes was analyzed (n > 10). The PBSs at day 0 were arranged densely on the membranes with a randomly distributed pattern (Fig. 4A), and the density of the PBSs was calculated to be 384 ± 17 PBS/μm^2 (Table 1). In response to nitrogen starvation, the PBS density on the membranes decreased gradually (Fig. 4B–E). The PBS density decreased by ~28% during the first 5 days, and after 10 days, the density was reduced to <50%. PBSs became sparse and lipid layers became exposed. At 20 days, the density of PBSs on the membranes decreased to only 51 PBS/μm^2 (Table 1).

Average size of the PBSs at each nitrogen starvation stage was calculated (n > 300). On day 0, the average size of the PBSs on the thylakoid membranes was 58 ± 4 nm (length), 40 ± 3 nm (width) and 27 ± 2 nm (height) (n = 300) (Table 1). The sizes of PBSs gradually decreased during nitrogen starvation. A decrease in the size of the PBSs was evident after 5 days, and the size reduced about 6 nm in length, 5 nm in width and 2 nm in height. Then, the size decreased slower from 5 to 20 days. The average dimensions of the PBSs were 49 ± 4 nm (length), 31 ± 3 nm (width) and 14 ± 2 nm (height) after 20 days (Table 1). After 20 days of nitrogen starvation, the size of the PBSs decreased approximately 15% in length, 23% in width and 48% in height, compared to the size at day 0. Although the PBSs decreased in size, the hemispherical morphology of the PBSs was retained during nitrogen starvation (Fig. S3).

Table 1
Changes in sizes of PBSs (n > 300) and PBS density on thylakoid membranes (n > 10) during nitrogen starvation ($p < 0.05$). Data are presented as means ± standard deviation.

Time (days)	Length (nm)	Width (nm)	Height (nm)	Density (μm^{-2})
0	58 ± 4	40 ± 3	27 ± 2	384 ± 17
5	52 ± 3	35 ± 3	25 ± 3	277 ± 18
10	51 ± 4	34 ± 3	20 ± 2	184 ± 23
15	49 ± 4	32 ± 3	19 ± 2	100 ± 33
20	49 ± 4	31 ± 3	14 ± 2	51 ± 15

During nitrogen starvation, holes appeared in the thylakoid membranes (Fig. 4). The holes were distributed throughout the membranes.

3.5. PBS-free thylakoid membranes

Thylakoid membranes on which PBSs were removed were imaged with AFM (Fig. 5). Results showed that most PBSs on thylakoid membranes were removed, and only a few PBSs could be occasionally recognized. High resolution images showed that there were only particles with diameters of 11–15 nm (Fig. S4), which were possibly to be photosystems or other complexes instead of PBSs. Compared with PBS-free thylakoid membranes, we consider that the large protein complexes we observed on native thylakoid membranes should be attributed to PBSs.

Holes could be noticed on PBS-free thylakoid membranes, like those on native thylakoid membranes. Sizes of small holes were similar to that of a PBS. Holes larger than the size of PBS could be

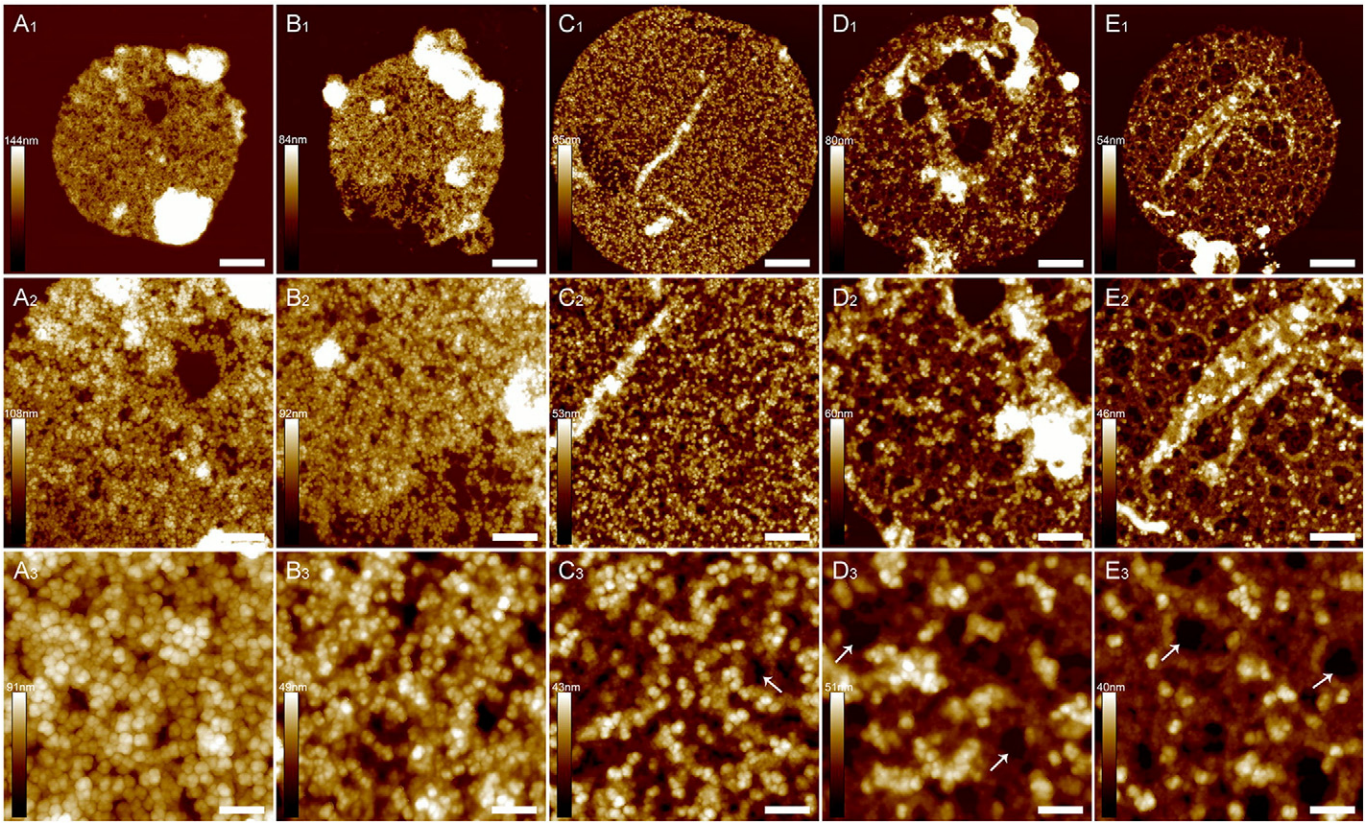

Fig. 4. AFM images of PBS-thylakoid membranes from *P. cruentum* grown under nitrogen starvation for 0 (A_1–A_3), 5 (B_1–B_3), 10 (C_1–C_3), 15 (D_1–D_3) and 20 days (E_1–E_3). (A_2–E_2), Enlarged AFM images from (A_1–E_1), respectively. (A_3–E_3), Enlarged AFM images from (A_2–E_2), respectively. Holes in the thylakoid membranes are indicated by arrows. Scale bar: A_1–E_1, 1 μm; A_2–E_2, 500 nm; A_3–E_3, 200 nm.

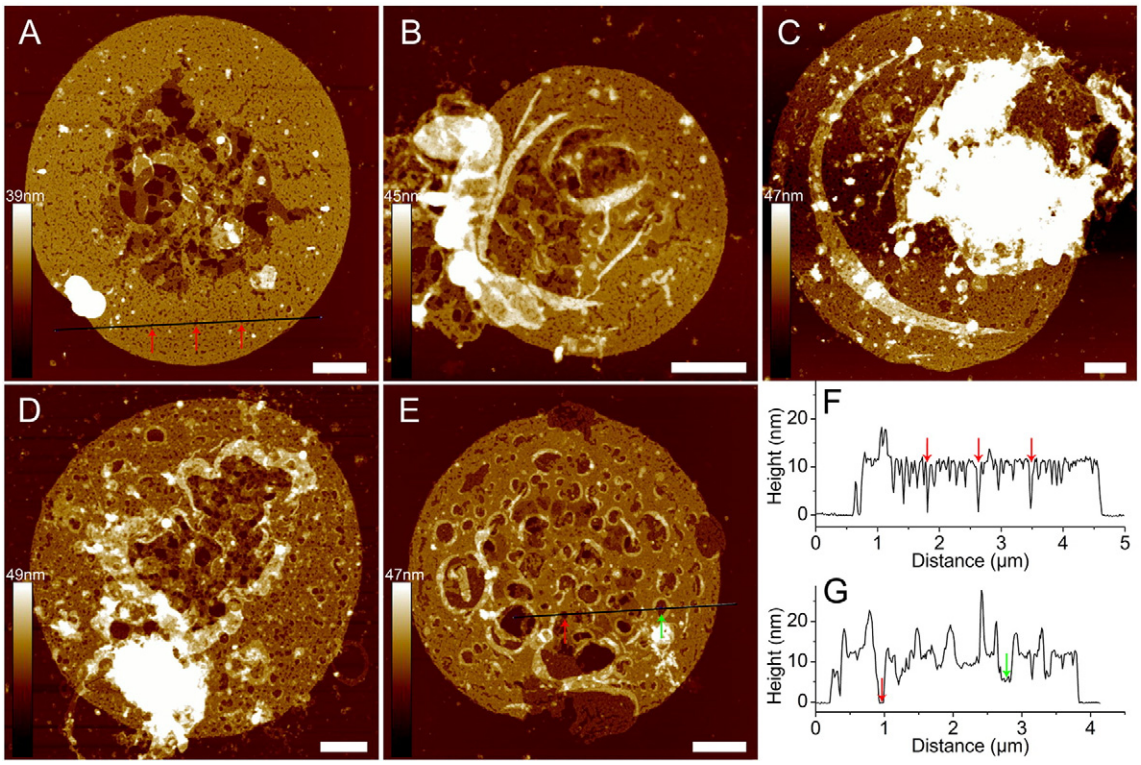

Fig. 5. AFM images of PBS-free thylakoid membranes (A–E) from *P. cruentum* grown under nitrogen starvation for 0 (A), 5 (B), 10 (C), 15 (D) and 20 days (E). Scale bar: 1 μm. (F), Height profile of panel (A). (G), Height profile of panel (E). The red and green arrows showed the height of holes. Lines indicated cross section positions.

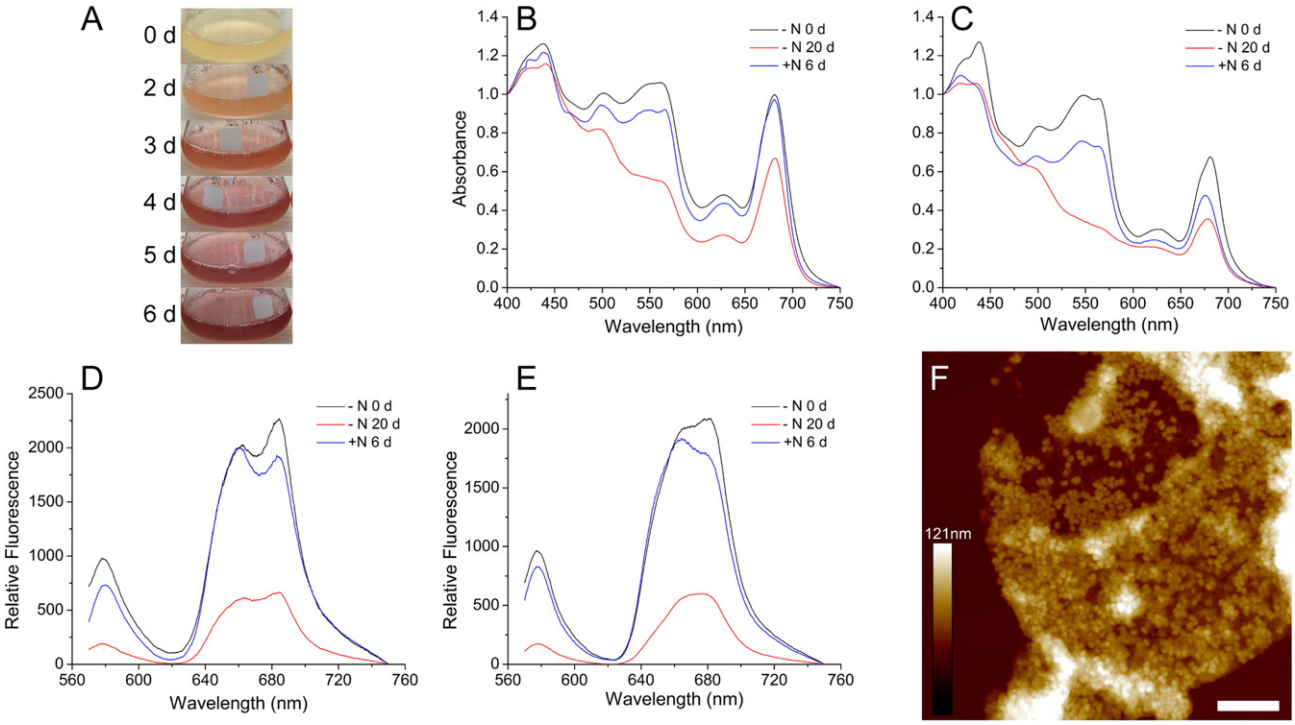

Fig. 6. Recovery of *P. cruentum* upon addition of nitrogen source. Photos of nitrogen recovered *P. cruentum* (A). Absorption spectra of nitrogen recovered cells (B) and PBS-thylakoid membranes (C). Room temperature fluorescence spectra of nitrogen recovered cells (D) and PBS-thylakoid membranes (E) excited at 545 nm. AFM images of nitrogen recovered PBS-thylakoid membranes for 6 days (F). Scale bar: 500 nm.

noticed, and the amount of the large holes tend to increase as the time of nitrogen starvation increased.

3.6. Recovery upon addition of nitrogen

Experiments on recovery upon addition of nitrogen was performed (Fig. 6). Nitrate was added to the 20-days nitrogen starvation culture. The color of the culture turned from yellow-green to dark red. The absorption spectra and the fluorescence emissions of phycobiliproteins in cells and isolated thylakoid membranes were recorded. The density of PBSs on the membrane was increased as observed by AFM. These results indicated that PBSs recovered after addition of nitrogen sources.

4. Discussion

AFM is a powerful tool for investigating supramolecular structures of membrane proteins with high resolution [19]. In recent years, AFM-based research on photosynthetic membranes from purple bacteria and higher plants has increased. However, direct observations of changes in the supramolecular architecture of photosynthetic membranes from any photosynthetic organism in response to nitrogen stress are still lacking. In our previous work, we reported on the supramolecular architecture of thylakoid membranes from red alga (*P. cruentum*) that was observed using AFM, and showed that AFM is useful for studying red algal thylakoid membranes [25]. Therefore, we used AFM as well as other techniques to investigate the supramolecular architectures of photosynthetic membranes from *P. cruentum* during nitrogen starvation.

Nitrogen starvation resulted in obvious changes in the structure of plastids from *P. cruentum*. The lamellae were reduced and distance between adjacent thylakoid membranes increased after 20 days of nitrogen starvation, indicating that the plastids were affected greatly by growth in a nitrogen-depleted environment. The thickness of the exopolysaccharide layers surrounding *P. cruentum* cells gradually increased. The amount of dark-staining inclusions increased, and they may be starch granules, as in previous reports [35]. The accumulation of glycogen in cyanobacteria during nitrogen starvation is well documented [36]. These results implied that the synthesis of polysaccharides in *P. cruentum* was more active after nitrogen starvation.

The overall morphology of the thylakoid membranes could not be observed from the images of the ultrathin sections, but they could easily be analyzed directly from the images of the isolated intact thylakoid membranes. In higher plants, most thylakoid membranes are connected to form stacked grana, and it is well documented that they are round vesicles. However, in red algae, where thylakoid membranes completely fill the plastids, much less research has been conducted. Gantt's group presented TEM images of an isolated round thylakoid vesicle with a diameter of <0.9 μm [37]. In our previous work, we presented TEM images of isolated thylakoid membranes, which were tube-like or irregular in shape [25,38]. In this work, we observed that all of the isolated thylakoid membranes were round vesicles, as observed in AFM images, except for some that were broken. Considering the possible limitations of AFM as well TEM, both round vesicles and tube-like/irregular vesicles might exist in the plastid of red algae. However, observation of isolated membrane vesicles could not give a satisfactory answer to the question of membrane conformation *in vivo*.

P. cruentum cultures gradually turned from red to yellow-green during nitrogen starvation. The bleaching in cyanobacteria or red algae that is caused by nitrogen stress is known as chlorosis [39]. The major red pigment in *P. cruentum* is PE, and the color change implies that PE undergoes degradation. The absorption spectra confirmed that phycobiliproteins were degraded gradually during nitrogen starvation. The absorbance of PE declined more dramatically than for other phycobiliproteins, indicating that the content of PE was reduced.

The supramolecular organization of thylakoid membranes was determined using AFM in order to study the changes in the size and density of PBSs. During nitrogen starvation, PBSs were observed to be arranged randomly on the thylakoid membranes. The density of PBSs was greatly affected by nitrogen starvation, and the reduction of PBS density was generally linear with time. Approximately 87% of the total PBSs were lost after 20 days. PBS size decreased with time, and PBSs decreased approximately 15% in length and 23% in width after 20 days of nitrogen starvation. PBSs of *P. cruentum* were composed mainly of PE (84%) and a small portion of R-PC (11%) and APC (5%) [40]. Moreover, PE is localized in the outer region of the PBSs. Thus, we suggest that a portion of the PE in PBSs was degraded during nitrogen starvation which resulted in a reduction of the size, and this was consistent with the absorption spectra data.

PBSs were distributed evenly on the native thylakoid membranes. During nitrogen starvation, the PBSs were degraded and the lipid layers underneath could be observed. After 15 days, larger areas of lipid layers were exposed, and most PBSs became aggregated to form clusters. This indicated that degradation of PBSs on thylakoid membranes was not completely random. After 20 days, when most of the PBSs were degraded, single PBSs remained on the thylakoid membranes.

Holes could be noticed on PBS-free thylakoid membranes and the native thylakoid membranes that were exposed during nitrogen starvation. Although it is not know yet how these holes were formed, there may be several possibilities for the formation of these holes. First, as small holes are of similar size to that of PBSs, they might result from loss of PBS. Larger holes might be formed by loss of an area of PBS or breakage of lipid membrane adjacent to the place where PBSs were lost. Second, since the heights of some holes were the same as that of the mica support, they might not really be holes on one membrane layer. Instead, they might possibly be perforations such as those observed in cyanobacterial thylakoid membranes [41]. Third, the possibility that holes were formed by sample preparation should not be excluded. How these holes were formed is not yet known based on present results, and further work is needed in the future.

High resolution images of PBS-free thylakoid membranes showed that there were many small particles densely arranged on the membranes. The sizes of these particles were about 11–15 nm in diameter. Considering these particles fall within the size range of the photosystems, they might possibly be attributed to photosystem I or photosystem II. The density of these particles on thylakoid membranes from *P. cruentum* cultured in medium with nitrate was about 2200–2800 particle/μm^2, which was much higher than the density of PBS. However, it was hard to distinguish the nature of these complexes directly with the AFM technique alone.

A deficiency of nitrogen would likely affect the metabolism of the cells. Although information concerning global regulation of the metabolic networks in red algae during nitrogen starvation is lacking, the expression of some enzymes is known to be regulated in red algae when the nitrogen source was depleted [42]. In cyanobacteria, NblA, NblR and other proteins are involved in PBS degradation [30,43], but the molecular mechanism of PBS degradation in red algae is still not clear. In red algae, Ycf18, the homolog of cyanobacterial NblA, seemed not to be involved in the degradation of PBSs [44]. Thus the enzymes and regulators that participate in the degradation of PBSs in red algae still need to be elucidated.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2016.08.005.

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