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Photosystem II protein clearance and FtsH function in the diatom *Thalassiosira pseudonana*

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Abstract All oxygenic photoautotrophs suffer photoinactivation of their Photosystem II complexes, at a rate driven by the instantaneous light level. To maintain photosynthesis, PsbA subunits are proteolytically removed from photoinactivated Photosystem II complexes, primarily by a membrane-bound FtsH protease. Diatoms thrive in environments with fluctuating light, such as coastal regions, in part because they enjoy a low susceptibility to photoinactivation of Photosystem II. In a coastal strain of the diatom *Thalassiosira pseudonana* growing across a range of light levels, active Photosystem II represents only about 42 % of the total Photosystem II protein, with the remainder attributable to photoinactivated Photosystem II awaiting recycling. The rate constant for removal of PsbA protein increases with growth light, in parallel with an increasing content of the FtsH protease relative to the substrate PsbA. An offshore strain of *Thalassiosira pseudonana*, originating from a more stable light environment, had a lower content of FtsH and slower rate constants for removal of PsbA. We used this data to generate the first estimates for in vivo proteolytic degradation of photoinactivated PsbA per FtsH$_6$ protease, at ~$3.9 \times 10^{-2}$ s$^{-1}$, which proved consistent across growth lights and across the onshore and offshore strains.

Keywords Diatom D1 D2 · FtsH · Photoinactivation · Photosystem II · PsbA PsbD

Introduction

All oxygenic photoautotrophs suffer photoinactivation of Photosystem II (Vass and Cser 2009), which they must counter through a repair cycle involving proteolytic removal of damaged subunits and reassembly of a functional PSII from recycled and new protein subunits and co-factors (Aro et al. 1993; Edelman and Mattoo 2008; Nixon et al. 2010; Komenda et al. 2012). Under changing light photoautotrophs must regulate the short- and long-term rates of the steps in the repair cycle (Edelman and Mattoo 2008). Photoinactivation is driven at a rate proportional to the instantaneous light level (Campbell and Tyystjärvi 2012; Schreiber and Klughammer 2013). If this photoinactivation outruns the repair process, the cells will suffer burn-down of their pool of functional PSII (Behrenfeld et al. 1998), which leads to loss of photosynthesis and eventually to cell death if unchecked. Conversely, excessive investment in the protein...
synthesis and degradation enzymatic systems above requirements imposes a metabolic burden on the phototroph. The mechanisms of these repair processes have been largely defined for unicellular cyanobacteria whose thylakoids are organized concentrically with the plasma membrane (Nixon et al. 2010; Komenda et al. 2012) and in some chlorophyll a/b plants, whose thylakoids are organized into stacked grana regions rich in PSII, and exposed stromal regions where PSII reassembly occurs (Aro et al. 1993; Goral et al. 2010; Chi et al. 2012). In both plants and cyanobacteria, the initial photoinactivation of PSII occurs through multiple paths whose relative importance depends upon the conditions and upon the physiological state of the organism (Oguchi et al. 2011). A complication is that many environmental or metabolic conditions which provoke increased photoinactivation also provoke ROS-related inhibitions of the counteracting PSII repair process (Nishiyama et al. 2006; Murata et al. 2007).

In cyanobacteria, PSII photoinactivation is thought to provoke a conformational change in the interactions among PSII subunits (Lindahl et al. 2000; Nixon et al. 2010; Komenda et al. 2012) that allows FtsH2/H3, a hexameric AAA zinc protease (Ito and Akiyama 2005) associated with the thylakoid surface (Lindahl et al. 2000; Wagner et al. 2012), to recognize a specific N-terminal region of the PsbA (D1) subunit of PSII. FtsH2/H3, then uses ATP hydrolysis (Lindahl et al. 2000; Bruckner et al. 2003; Nixon et al. 2005) to pull PsbA into a central proteolytic chamber where the protein is processively cut into peptides. In cyanobacteria, PSII can migrate laterally through the thylakoids (Mullineaux 1999) and PSII processing may be localized to specific regions of the cyanobacterial thylakoids (Komenda et al. 2012). In chl a/b organisms with stacked thylakoids, protein subunits of photoinactivated PSII are phosphorylated (Koivuniemi et al. 1995), which appears to stimulate a lateral migration to either a grana margin or a stromal region of the thylakoid (Goral et al. 2010), where photoinactivated PSII disassembles into sub-complexes to allow the establishment of the FtsH2/H3/PSII interaction (Yoshioka et al. 2012). Mutant cyanobacteria lacking FtsH protease can still survive, but only under low to moderate light using alternate, lower capacity proteolytic paths to clear photoinactivated PSII (Komenda et al. 2012).

The FtsH2/H3 mediation of PsbA protein proteolysis has been established through mutational studies of both FtsH and the PsbA binding sequence (Komenda et al. 2006; Komenda et al. 2007), but the stoichiometries between FtsH2/H3 and PSII complexes, and the in vivo processing rates of FtsH2/H3 acting upon PsbA are as yet undefined, a gap we herein seek to fill.

Marine diatoms account for ~20% of global productivity (Field et al. 1998; Armbrust 2009; Falkowski 2012) and particularly thrive in areas of vertical water mixing (MacIntyre et al. 2000) where they exploit a variable light environment, which imposes rapid fluctuations in the rate of photoinactivation of PSII. These diatoms use a complex and extensive regulation of non-photochemical quenching of excitation (Lavaud et al. 2004, 2007; Bailleul et al. 2010; Zhu and Green 2010; Wu et al. 2012) to cope with fluctuating light, and their capacities for induction of non-photochemical quenching of excitation correlate with the light variability of their particular habitats (Lavaud et al. 2007; Bailleul et al. 2010). Furthermore, under low to moderate light marine centric diatoms show a low intrinsic susceptibility to photoinactivation of PSII (Key et al. 2010; Wu et al. 2011) in comparison to other, competing phytoplankton taxa (Six et al. 2007, 2009; Ragni et al. 2008; Loebl et al. 2010; McCarthy et al. 2012).

Aside from their ecological and biogeochemical importance, the diatoms are photophysiological intriguing. Their chlorophyll a/c chloroplasts house thylakoids arranged in loosely stacked triply layered bands (Gibbs 1962; Drum 1963; Lepeit et al. 2012) that lack differentiation into grana and stromal regions, yet appear more closely associated than the concentric thylakoids of cyanobacteria, which are spaced by the large extrinsic phycobilisome antenna complexes (Mullineaux 1999). This raises the question of how the surface-associated FtsH6 protease hexamer can access photoinactivated PSII (Komenda et al. 2012), without apparent regions of exposed thylakoid. Furthermore, although the subunit composition and subunit sequences of PSII are highly conserved across photoautotrophs, the PSII complexes of diatoms do present some particularities in PSII subunit composition (Nagao et al. 2010) which may relate to their distinct photophysiological properties (Grouneva et al. 2012). Isolated diatom thylakoids contain three metalloproteases, one of which might correspond to FtsH, and also a serine protease, all associated with a chlorophyll a/c fucoxanthin binding protein (Nagao et al. 2012). These membrane preparations suffer slow loss of PsbA and PsbD proteins (Nagao et al. 2012) over 3–9 h, in the absence of ATP. As FtsH requires ATP for proteolysis, this slow loss apparently represents an alternate path(s) for removal of PsbA and PsbD from diatom thylakoids.

We have now assembled a nearly comprehensive coverage of the in vivo pool sizes and rate constants for the key sub-processes of the PSII repair cycle in the model coastal diatom Thalassiosira pseudonana CCMP 1335, growing across a range of light. Surprisingly, in the related off shore strain Thalassiosira pseudonana CCMP 1014 the PsbD (D2) and PsbB (CP47) subunits of PSII degrade with rates and magnitudes comparable to PsbA (Wu et al. 2011, 2012), suggesting that diatom PSII protein degradation (Nagao et al. 2012) shows distinct properties from model cyanobacteria (Yao et al. 2012). These distinctive properties may relate to the particular NPQ mechanisms and
exploitation of variable light in diatoms. In parallel, we quantified the total cellular content of FtsH$_6$ protease to place the first published boundary constraints on the in vivo performance of FtsH in PSII subunit processing in a photosynthetic organism.

**Materials and methods**

**Culture protocol**

The coastal centric diatom, *Thalassiosira pseudonana* (Hustedt) Halse et Heimdal obtained from the Provasoli-Guillard National Center of Marine Phytoplankton (CCMP 1335) was cultured in 2-cm-thick cuvettes (450 mL volume) of FMT-150 photobioreactors (Photon Systems Instruments, Drasov, Czech Republic) at 18°C in enriched artificial seawater (EASW) prepared according to Berges et al. (2001), except with 54.5 μM Si and 0.82 μM Sr. Cultures were gently mixed by a curtain of bubbles emitted from four apertures across the cuvette bottom with air at ambient (~390 ppmv) pCO$_2$. Before bubbling into the bioreactor culture cuvette, the air streams were filtered through a 0.2 μm micro-filter and bubbled through sterile distilled water for humidification. The growth of *T. pseudonana* was not notably disrupted by this bubbling (Shi et al. 2009). The offshore strain *Thalassiosira pseudonana* CCMP 1014), also obtained from the Provasoli-Guillard National Center of Marine, was grown in dilute batch cultures as described in Wu et al. (2011, 2012).

Continuous growth light was measured with a micro-spherical quantum sensor (US-SQS, Waltz, Germany). For *Thalassiosira pseudonana* CCMP 1335 turbidostat cultures, the light intensities in culture vessels filled with seawater were set to ~30, 80, 160, 240, or 380 μmol photons m$^{-2}$ s$^{-1}$, provided by a panel of blue LED (450 nm) covering the entire rear face of the cuvette. These light levels approximate a range from near the bottom of the euphotic zone at 1.5% of surface sunlight, up to the upper 35% of the euphotic zone in marine ecosystems. Note that the blue light provokes a higher ratio of PSII photoinactivation:photochemistry than does white light (Wu et al. 2011), and that high white light supports higher maximum growth rates, under high levels of PAR, than our blue light source (Cullen and Lewis 1988). We chose the blue light environment to (roughly) approximate the blue bias of marine light fields, and to ensure that our measures of the effective absorbance cross-sections for photoinactivation matched the growth light regime for the cultures.

**Growth rate**

We obtained a preliminary growth curve by operating the bioreactors in batch mode to determine a proper cell density set-point for turbidostat mode that maintained a stable pH (indicating stable DIC status) and a detectable $F_0$ fluorescence signal from the diatoms, monitored continuously using the onboard photodiode sensor in the bioreactors (Li and Campbell 2013). We then grew 4–6 replicate cultures for each light level by re-inoculating cells after cleaning and autoclaving the photobioreactor cuvettes. We grew each experimental replicate culture from initial inoculation for 3–4 generations without dilution until the culture density reached the set-point of basal fluorescence value ($F_0 = 280; 62 ± 30$ ng Chl a mL$^{-1}$). We then entered turbidostat mode to maintain this cell density by activating a peristaltic pump when the $F_0$ value reached the set-point to dilute the 450 mL culture with a 10% volumetric addition of media delivered from a reservoir, which was continuously pre-bubbled with the air stream to pre-equilibrate the dissolved inorganic carbon system of the media before addition to the culture volume. 3.5 L of media in the reservoir supported 9.5 cell generations of growth for each replicate culture, before we harvested for the light shift experiment, analyses of PSII content and biochemical analyses are described below. The temporal duration of each turbidostat run varied between 160 and 200 h, depending upon the achieved growth rate of the culture, which in turn varied with the applied growth light. At the end of the turbidostat growth period, we measured the cell volume and cell suspension density with a Multi-sizer 3 Counter (Beckman Coulter Inc., USA).

**Photoinactivation ($\sigma_i$) determination**

At the end of the turbidostat growth period, we took a 280 mL culture sample from each replicate for determination of the functional absorption cross-section for photons driving PSII photoinactivation ($\sigma_i$, A$^2$ quanta$^{-1}$) (Oliver et al. 2003; Six et al. 2007; Key et al. 2010; Campbell and Tyystjärvi 2012). We divided the culture sample into two flasks, and supplemented one with a final concentration of 500 μg mL$^{-1}$ lincomycin (Sigma-Aldrich) to inhibit chloroplast ribosome function (Key et al. 2010; Wu et al. 2011; McCarthy et al. 2012), thereby blocking PSII repair. We placed both the flasks in the dark for 10 min to allow the antibiotic (if present) to penetrate into cells and inhibit ribosome function. We then shifted the flasks to 450 μmol photons m$^{-2}$ s$^{-1}$ blue light (LEE #183, Panavision; peak transmission at 455–479 nm and 50% transmission at 406–529 nm, approximating the growth light quality in the bioreactors). In this way, we aimed to assess the responses of cells acclimated to a range of growth light levels to a shift to higher light, simulating a mixing event to the upper region of the euphotic zone. After 15-, 30-, 60-, and 90-min exposure to 450 μmol photons m$^{-2}$ s$^{-1}$, we took samples from both flasks for chlorophyll fluorescence
measurements. After 90 min, we returned the cultures to their culture growth light level and tracked any recovery over 60 min. We estimated the susceptibility of PSII to photoinactivation ($\sigma_i$, $A^2$ quanta$^{-1}$) by fitting the exponential decrease of PSII photochemical yield ($F_v/F_M$) of the lincomycin-treated sample versus cumulative incident photons per area. To obtain $F_v/F_M$, a 2 mL sample from each culture replicate of each time point was dark adapted for 5 min in a temperature-controlled cuvette holder (18 °C). We applied a blue-green modulated measuring light (4 Hz; Xenon-PAM, Waltz, Effeltrich, Germany) to measure $F_v$ with a saturating light pulse (4,000 µmol photons m$^{-2}$ s$^{-1}$, 600 ms) to measure the dark-adapted maximal fluorescence ($F_M$), and calculated $F_v/F_M$:

$$F_v/F_M = (F_M - F_0)/F_M$$

During the growth light recovery period, we continued to monitor $F_v/F_M$. We attributed any increase in $F_v/F_M$ in the lincomycin-treated cells to slow relaxation (Wu et al. 2012) of non-photochemical quenching, and used the amplitude of any relaxation to correct for the influence of non-photochemical quenching on changes in the measured $F_v/F_M$. Such corrections were small to negligible and had no substantive influence on the results. The data underlying the estimations of $\sigma_i$ have been deposited in our open database phytoplankton.mta.ca.

Oxygen flash yield quantitation of the content of active PSII

Before and after light shift treatments, we followed the methods published in Wu et al. (2012) to estimate the content of functional, active PSII centers in the cultures, following the oxygen flash yield approach of Chow et al. (1989, 2012). From the same culture samples, we then extracted total chlorophyll into 90 % acetone, measured absorbance, and used the equations of Jeffrey and Humphrey (1975) to estimate the chlorophyll $a$ content of the concentrated culture suspensions:

$$[\text{mg Chl a L}^{-1}] = 11.47 \times (A_{664} - A_{750}) - 0.4 \times (A_{630} - A_{750}).$$

Protein measurements

Just prior to the high light shift experiments (T0 samples) or after the 90 min of high light treatment, both with or without lincomycin (T90 samples), we vacuum-filtered 50 mL of culture onto a binder-free Whatman GF/F glass fiber filter (25 mm in diameter), which we immediately flash froze in liquid nitrogen and stored at −80 °C until later analyses of protein and chlorophyll, following Wu et al. (2011) to estimate the molar levels of PsbA (www.agrisera.se, antibody AS05084; standard: AS01016S), PsbD (www.agrisera.se, antibody AS06146; standard: AS09146S), and FtsH (www.agrisera.se, antibody AS11 1789; standard: new product) per µg total protein using quantitative immunoblotting (Six et al. 2007; Brown et al. 2008). We used Novex MagicMark™ XP Western Protein Standard (Life Technologies) to calibrate the molecular weight of detected bands.

To measure the clearance of PsbA and PsbD proteins, we plotted the data for fmol PsbA µg protein$^{-1}$ or fmol PsbD µg protein$^{-1}$ versus time for cultures treated with lincomycin to block chloroplastic protein synthesis, and then fit exponential decay functions to extract first order rate constants for the clearance of PsbA and PsbD (Wu et al. 2011, 2012).

Data analysis

We used ANOVA with Bonferroni post-tests (Prism 5, GraphPad Software) and comparisons of linear and non-linear curve fits to detect significant differences among the culture-light treatments.

Results and discussion

Thalassiosira maintains similar pools of active PSII across a wide range of growth lights

We estimated the fmol active PSII µg protein$^{-1}$ in Thalassiosira pseudonana CCMP 1335 for turbidostat cultures growing across a range of light levels. We used the approach of Chow et al. (1989, 2012) by driving oxygen evolution using a train of single turnover, saturating flashes sufficient to provoke a single charge separation through each active PSII, for each flash. From the same samples taken from the turbidostat cultures, we assayed total chlorophyll $a$ and total protein content, and used the gross oxygen evolution rate to estimate the number of active PSII per µg total protein (Fig. 1a). We found 39 ± 10 (SD) fmol PSII active µg protein$^{-1}$, with no significant trend with growth light. From the same culture samples, we used quantitative immunoblotting to determine fmol PsbA µg protein$^{-1}$ (Li and Campbell 2013) and in Fig. 1b, we plot the ratio of PSII active PsbA$^{-1}$, showing an average of 0.42 PSII active PsbA$^{-1}$ with again no significant trend with growth light. Estimating the ratio of PSII active PsbD$^{-1}$ gave similar results (data not presented). Thus, on a protein basis, the cultures maintained a stable pool of PSII active across an 11-fold range of growth lights.

Estimating rate constants for the PSII repair cycle

We treated cultures with lincomycin to block the synthesis of chloroplast proteins, and thereby block the repair of
PSII, incubated them under 450 μmol photons m⁻² s⁻¹ and then determined their susceptibility to photoinactivation with cumulative photon exposure using a target size formulation $r_i$ (Å² quanta⁻¹). Following Campbell and Tyystjärvi (2012), for growth light level I, the rate constant for photoinactivation, $k_{pi} = r_i I$.

Using immunoquantitations on samples frozen from the same lincomycin time course experiments, we estimated experimental rate constants for removal of PsbA, $k_{psbA}$ and PsbD, $k_{psbD}$. Our formulations of rate equations are given in “Appendix.” The upward light shift to 450 μmol photons m⁻² s⁻¹ is sufficient to saturate the short-term removal rates for PsbA and PsbD in *Thalassiosira pseudonana* across the range of growth lights we used (Wu et al. 2011, 2012). These rate constants thus reflect the rapidly inducible capacity to remove the proteins in cells at the given growth lights. Our cultures were in balanced growth (Li and Campbell 2013) with steady pools of functional PSII (Fig. 1), so the actual removal rates for PsbA and PsbD prevailing at a particular growth light would be slower, in balance with the slower rates of photoinactivation under lower growth light. The experimental rate constants $k_{psbA}$ and $k_{psbD}$ are estimated as loss of the protein from the total cellular pool of the PsbA or PsbD protein, as detected by binding of a specific antibody. They are kinetically valid, but mechanistic studies of the PSII repair cycle (Nixon et al. 2010; Komenda et al. 2012), and our own kinetic modeling (data not presented), show that inactivated PsbA is actually removed principally from the sub-population of PSII that has been photoinactivated. That is, the removal process discriminates between inactivated and active PSII. As shown in Fig. 1b, only a fraction of the total PsbA pool is found in PSIIactive, with the remainder largely attributable to the PSIIinactive pool. Furthermore, progressive protein aggregation or oxidative changes to amino acids could potentially interfere with the binding of our antibody to the target protein, thereby leading to an over-estimate of protein loss from the cells.

In Fig. 2a, we plot $k_{pi}$ (closed symbols) and $k_{psbA}$ (open symbols) versus growth light. Over the range of moderate growth lights $k_{pi}$ scales linearly with growth light. $k_{psbA}$ also shows a positive correlation with growth light ($F$ test, $P = 0.0204$), so cells growing under higher light have a significantly higher maximum short-term capacity to clear PsbA protein. The acclimation of $k_{psbA}$ with increasing growth light does not, however, keep pace with the significant increase in $k_{pi}$ ($F$ test, $P < 0.0001$) so under the highest growth lights the rate constants converge. Figure 2b shows the parallel determinations of $k_{psbD}$ rate constants for the clearance of PsbD, which are comparable in magnitude to the rate constants for clearance of PsbA, at least over the short-term lincomycin treatments of 90 min under 450 μmol photons m⁻² s⁻¹ applied. $k_{psbD}$, however, showed no significant trend with increasing growth light.

**Thalassiosira** FtsH protease quantitation and function in vivo

We collaborated with AgriSera AB (www.agrisera.se) to create a global antibody that reacts with a peptide target conserved across chloroplast FtsH protease subunits, and then contracted with Environmental Proteomics NB (www.environmentalproteomics.ca) to create an accompanying quantitation standard produced by over-expressing
the FtsH2 isoform, encoded by gene slr0228, from *Synechocystis* PCC 6803. We used the antibody and standard to quantify a pool of FtsH monomers from *Thalassiosira pseudonana* CCMP 1335 (Figs. 3, 4a) comprised of the chloroplast-encoded FtsH protein isoform (ThpsCp046, The Stramenopile Chloroplast Genomics Project; http://chloroplast.ocean.washington.edu/) along with three related nuclear encoded isoforms in the *Thalassiosira pseudonana* (Armbrust et al. 2004) genome. Our quantitation of these FtsH proteins from *Thalassiosira pseudonana* places an upper bound upon the pool of chloroplastic FtsH potentially available to act upon PsbA protein. We find that as the growth light for cultures of the onshore strain *Thalassiosira pseudonana* increases, the ratio of FtsH 6:PsbA (Fig. 4b, closed symbols) increases significantly (*F* test, *P* < 0.0001), showing acclimation of the cellular proteolytic apparatus to increased growth light. In contrast, in samples taken from dilute batch cultures of the offshore strain *Thalassiosira pseudonana* CCMP 1014 (Wu et al. 2012) the ratio of FtsH6:PsbA (Fig. 4b, open symbols) is lower, and we did not find an increase with increasing growth light. Note that our global antibody will detect the chloroplastic isoform of FtsH, but also three related nuclear encoded FtsH isoforms. Therefore, the true chloroplastic pool of FtsH6 mediating PsbA degradation may be smaller (to some degree) than our quantitation of FtsH subunits. We suspect, however, that in these diatoms the chloroplastic FtsH pool dominated our detections, because diatoms growing under high light showed higher content of FtsH, and because our measured PsbA degradation rates correlate well with FtsH content (Fig. 4C).

**Fig. 2** The rate constants for photoinactivation of PSII, *k*π (s−1) (closed circles) at the given culture growth light, and for maximum short-term clearance of PsbA, *k*psbA (s−1) (open circles) (a) or PsbD, *k*psbd (s−1) (open triangles) (b) plotted versus culture growth light. Each point represents a separate experimental determination based upon a light shift experiment on a separate turbidostat culture, treated with lincomycin and then incubated under light high enough to saturate the removal rates for PsbA and PsbD. Dotted lines 95% confidence intervals on the linear regressions.

**Fig. 3** Quantitative immunodetection of FtsH protein from *Thalassiosira pseudonana*. Total protein was extracted from samples from multiple separate cultures of *Thalassiosira pseudonana* CCMP 1335 grown under a range of light levels from 30 to 380 μmol photons m−2 s−1. 6 μg of total protein extract was loaded in sample lanes 7–16. The leftmost lane 1 contains MagicMark XP protein standard that reacts with the chemiluminescent detection system to give visible bands, marked with their size in kDa. Lanes 2–6 contain a series of decreasing loads of quantitated FtsH protein standard generated by overexpression of the gene slr0228, from *Synechocystis* PCC 6803. The chemiluminescent signal was captured using a BioRad Versadoc 4000 CCD imager. The FtsH signal from the sample bands was quantified by comparison to the quantitation standard curve. The expected size of the *Thalassiosira* FtsH protein based upon its sequence is 70.2 kDa, consistent with migration just below the 80 kDa molecular weight marker.

The N-terminal 20 amino acids of PsbA are important for rapid clearance of PsbA protein from *Synechocystis* PCC 6803 (Komenda et al. 2007), and deletion of these amino acids generated a phenotype similar to the phenotype for deletion of the FtsH2 isoform, encoded by the gene.
slr0228, from *Synechocystis* (Komenda et al. 2006). These N-terminal 20 amino acids allow the FtsH23H33 hexameric protease to recognize PsbA after it is exposed by photoinactivation of PSII. Our preliminary kinetic modeling of PsbA and PSII dynamics in *Thalassiosira pseudonana* CCMP 1014 and 1335 indicate that, as in *Synechocystis* (Komenda et al. 2007), removal of PsbA protein occurs after PSII photoinactivation. That is, the substrate for FtsH-mediated removal of PsbA is photoinactivated PSII, not the total pool of PSII. We aligned the N-terminal regions of the PsbA and PsbD proteins from *Thalassiosira pseudonana* and from *Synechocystis* PCC 6803. As expected, the PsbA cluster with each other, as do the PsbD proteins.

MIATLERREG VSLWERFCAW ITSTENRLYI MTTTLQQRES ASLWEQFCQW VTSTNNRIYV

MTIAIGQNQ- ERGLFDLVDD WLKRDRFVFI
MTIAVGRAPV ERGWFDVLDD WLKRDRFVFI

Over the N-terminal 20 residues, the PsbA sequences from *Thalassiosira pseudonana* and *Synechocystis* are identical at 12 positions, supporting the functional conservation of this region in PsbA turnover. Thus, both sequence conservation with *Synechocystis*, and the strong correlation between $k_{\text{psbA}}$ and FtsH$_6$:PsbA in *Thalassiosira* (Fig. 4C) support FtsH-mediated removal of PsbA protein, with increases in FtsH content mediating faster removal of PsbA under increasing growth light.

In *Synechocystis* PCC 6803, the PsbA proteins turn over ~5× faster than the related PsbD protein that forms the other half of the PSII core (Yao et al. 2012). In contrast, in *Chaetoceros gracilis* (Nagao et al. 2012), in *Thalassiosira pseudonana* CCMP 1014 (Wu et al. 2011, 2012), and now in *Thalassiosira pseudonana* CCMP 1335 (Fig. 2b), the rate constant $k_{\text{psbD}}$ for removal of PsbD is comparable in magnitude to $k_{\text{psbA}}$ for removal of PsbA. There is no clear conservation of the critical N-terminal 20 amino acids
between PsbA and PsbD, in *Thalassiosira* or in *Synechocystis*. The removal of PsbD is high but variable, and in contrast to PsbA, shows no correlation with increasing growth light; indeed some of our highest estimates for *k*$_{\text{psbD}}$ came from cells growing under low light. *k*$_{\text{psbD}}$ does not correlate with FtsH$_6$:PsbD in *Thalassiosira*, so either the FtsH content of these cells under low light is already sufficient to maximize the capacity for removal of PsbD (but not PsbA; Fig. 2a), or, PsbD is cleared through a path that is not directly limited by FtsH content. We thus hypothesize that the high, but variable (Fig. 2b), rate constants for removal of PsbD result from a different path, consistent with evidence that PsbD degradation may be mediated by DegP (Chi et al. 2012), and that PsbD is cleared from isolated diatom thylakoids, possibly by one of the proteases associated with fucoxanthin-chlorophyll protein complexes (Nagao et al. 2012) that appear to function without a requirement for ATP.

In *Thalassiosira pseudonana* CCMP 1335, the rate constant for removal of photoinactivated PsbA protein, *k*$_{\text{psbA}}$, increased from $2.4 \times 10^{-3}$ s$^{-1}$ (SEM $2.6 \times 10^{-5}$) to $4.1 \times 10^{-3}$ s$^{-1}$ (SEM $2.8 \times 10^{-5}$) as growth light increased from 35 to 380 μmol photons m$^{-2}$ s$^{-1}$. This increase in the capacity to remove photoactivated PsbA protein allowed the cells to maintain their ratio of pools of PSII$_{\text{active}}$ versus PSII$_{\text{inactive}}$ (Fig. 1a) in the face of increasing photoactivation rates (Fig. 2a). In parallel, the content of the FtsH$_6$ protease, which mediates proteolytic removal of PsbA, increased from $3.3 \times 10^{-3}$ (SEM $2.2 \times 10^{-4}$) FtsH$_6$ hexamers per PsbA under the low growth light of 35 μmol photons m$^{-2}$ s$^{-1}$, up to $5.7 \times 10^{-3}$ (SEM $3 \times 10^{-4}$) FtsH$_6$ hexamers per PsbA under the super-saturating 380 μmol photons m$^{-2}$ s$^{-1}$ (Fig. 4b, closed symbols). Cells from high light growth have more FtsH$_6$ hexamers per PsbA and in parallel can achieve higher rate constants for removal of photoactivated PsbA (Fig. 4c, closed symbols). Again, the offshore strain *Thalassiosira pseudonana* CCMP 1014 shows both a lower ratio of FtsH$_6$:PsbA and a lower *k*$_{\text{psbA}}$ (Fig. 4c; open symbols). For both strains *k*$_{\text{psbA}}'$ versus FtsH$_6$:PsbA fell along a common regression line. Comparing these FtsH$_6$ and PsbA contents with the rate constants for PsbA removal places a lower limit on the achieved in vivo proteolytic turnover, or *k*$_{\text{cat}}$ of photoactivated PsbA per FtsH$_6$ protease in these diatoms of $3.9 \times 10^{-2}$ s$^{-1}$. This estimate for the in vivo *k*$_{\text{cat}}$ was consistent across the two strains growing under growth lights from 30 to 380 μmol photons m$^{-2}$ s$^{-1}$. Thus, with increasing growth light, or in the comparison of onshore to offshore strains, higher PsbA removal capacity results from the increase in the ratio of FtsH$_6$ to PsbA substrate, not from increased catalytic performance of the FtsH$_6$ complexes.

*Escherichia coli* contains FtsH$_6$ protease and an in vitro estimate of its catalytic rate constant for proteolysis, *k*$_{\text{cat}}$ (Bruckner et al. 2003) is slow, at 0.035 s$^{-1}$ per cut. If we assume that the chloroplast FtsH$_6$ protease displays the same preference for cutting after phenylalanine residues as does the *E. coli* FtsH$_6$ protease, then the 360 amino acid PsbA protein contains 30 such cut sites, and FtsH$_6$ processing progressively releases peptides of $\sim$27 amino acids into the chloroplast stroma. At 0.035 proteolytic cuts s$^{-1}$ this would imply a *k*$_{\text{cat}}$ for PsbA processing of 0.0012 s$^{-1}$. Our in vivo *k*$_{\text{cat}}$ for the diatom FtsH$_6$ of 0.04 s$^{-1}$ (SEM 0.003) is thus 33× faster than the in vitro proteolytic estimates for the *E. coli* enzyme. On the other hand, in vitro assays of ATP hydrolysis by FtsH$_6$, in the absence of peptide substrate, give much higher *k*$_{\text{cat}}$ values of 22 ATP s$^{-1}$ FtsH$_6$ (Bruckner et al. 2003). As 6–8 ATP hydrolyses accompany each proteolytic cut, this would imply a potential *k*$_{\text{cat}}$ of $\sim$3.7 proteolytic cuts s$^{-1}$ and a *k*$_{\text{cat}}$ for PsbA processing of $\sim$0.12 s$^{-1}$. Our in vivo *k*$_{\text{cat}}$ estimate of $\sim$0.04 s$^{-1}$ based upon in vivo measures of PsbA clearance and upon immunquantitation of in vivo FtsH content thus falls faster than estimates based upon in vitro proteolysis, and somewhat slower than estimates based upon in vitro ATP hydrolysis. To our knowledge, our estimates are the first approach to constrain the achieved in vivo proteolytic degradation rates for FtsH in a photosynthetic organism.

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Appendix: Rate equations

We formulated three rate equations describing the PSI repair cycle, to extract a mechanistic rate constant, *k*$_{\text{psbA}}'$, based upon removal of inactivated PsbA from the subpopulation of PsbA protein held in photoactivated PSI centers, rather than from the larger total pools of PsbA.

Our equations describe changes in the sizes of the three key pools of the PSI repair cycle:

\[ [\text{PSII}_{\text{total}}] = [\text{PSII}_{\text{activepq}}] + [\text{PSII}_{\text{inactive}}] + [\text{PSII}_{\text{diss}}] \]

Under balanced growth these pool sizes are steady, so the rate equation for change in each pool will sum to zero.

1. [PSII$_{\text{activepq}}$], the pool of active PSI, which under illumination will be under some degree of net down-regulation by non-photochemical quenching.
d[PSII\textsubscript{activenpq}]/dt = \{[PSII\textsubscript{inactive}] \times k_\textsubscript{psbA}'\}
+ \{([PSII\textsubscript{activenpq}] + [PSII\textsubscript{inactive}]) \times \mu\}
- \{[PSII\textsubscript{activenpq}] \times (k_\textsubscript{pi} + \mu)\}
\text{(RateEquation1)}

or, equivalently:

\begin{equation}
\frac{d[PSII\textsubscript{activenpq}]}{dt} = \{([PSII\textsubscript{activenpq}] + [PSII\textsubscript{inactive}]) \times (k_\textsubscript{psbA}' + \mu)\}
- \{[PSII\textsubscript{activenpq}] \times (k_\textsubscript{pi} + \mu)\}
\end{equation}

We assume the synthesis of PsbA is the rate-limiting step for reassembly of [PSII\textsubscript{activenpq}], since PsbA synthesis and PSII reassembly are mechanistically linked and co-regulating (Aro et al. 1993; Edelman and Mattoo 2008; Nixon et al. 2010; Komenda et al. 2012). Under balanced growth the total pool of [PsbA] is steady, and is distributed across the pools [PSII\textsubscript{activenpq}] and [PSII\textsubscript{inactive}], as [PSII\textsubscript{diss}] represents the PSII-sub-complexes which have been disassembled after removal of PsbA. Newly synthesized PsbA initially enters the pool of [PSII\textsubscript{activenpq}], at a rate sufficient to counter the clearance of PsbA is from [PSII inactive]. Experimentally, we measure the algebraically equivalent (see below) loss of PsbA from the total pool of PsbA as ([PsbA] \times k_\textsubscript{psbA}), equal to \{([PSII\textsubscript{activenpq}] + [PSII\textsubscript{inactive}]) \times k_\textsubscript{psbA}\}. For balanced exponential growth each macromolecular pool in the cell accumulates at the same exponential rate, \(\mu\), but the pools are subject to dilution by the same exponential growth rate, so that each daughter cell has the same composition as the mother cell. Thus, for each of our rate equations, we use \(\mu\) to contribute a dilution factor drawing upon each PSII sub-pool. Under high light the growth rate is small relative to the rate of photoinactivation of PSII, hence it is neglected in the family of models (e.g., Oliver et al. 2003) flowing from the classic Kok (1956) formulation of short-term photoinactivation and recovery of PSII. In diatom cultures, particularly under moderate light, growth is rapid while photoinactivation is fairly slow and so the growth rate constant is comparable in magnitude to the rate constant \(k_\text{pi}\) for PSII photoinactivation, and growth rate exerts a significant influence on the pools of PSII repair cycle intermediates (Cullen and Lewis 1988). We estimate our pool sizes on the basis of \(\mu\) total protein, and superficially, this normalization should remove the influence of growth on the PSII repair cycle, as the underlying growth rate increases the pool sizes with new molecules at the same rate that cell division dilutes the pools. However, to maintain balanced growth [PSII\textsubscript{total}] has to accumulate at the cellular growth rate \(\mu\), but newly synthesized PSII subunits (aside from PsbA) will enter the pool of [PSII\textsubscript{diss}] at rate \((\mu \times [PSII\textsubscript{total}])\) whereas growth dilution acts upon all three pools, [PSII\textsubscript{activenpq}], [PSII\textsubscript{inactive}], and [PSII\textsubscript{diss}] proportionally to their relative abundances. Thus, in the absence of PSII repair cycling, biosynthesis would continually bias the [PSII\textsubscript{total}] pool towards an increasing contribution of [PSII\textsubscript{diss}]. As the PSII repair cycle rate constants are comparable in magnitude to \(\mu\) under low growth light, the biosynthetic accumulation of PSII subunits is a significant and even dominant influence on PSII dynamics (Cullen and Lewis 1988), as opposed to photoinactivation or the counteracting clearance of photoactivated subunits which come to predominate under excess light. In our experiments under low to moderately high light, total non-photochemical quenching was low and did not change markedly in magnitude during our treatments, although during our high light shift experiments there was a shift from rapidly reversible NPQd to a more sustained, slowly relaxing NPQs (Zhu and Green 2010; Wu et al. 2012).

2. [PSII\textsubscript{inactive}], the pool of photoactivated PSII that still contains PsbA and PsbD but which is not photochemically active, having suffered irreversible damage to a protein subunit.

\begin{equation}
\frac{d[PSII\textsubscript{inactive}]}{dt} = \{[PSII\textsubscript{active}] \times k_\textsubscript{pi}\}
- \{[PSII\textsubscript{inactive}] \times (k_\textsubscript{psbA}' + \mu)\}
\end{equation}

This formulation assumes that the conversion of PSII\textsubscript{inactive} to disassembled subunits is limited by the removal of PsbA; an equivalent equation can be written if removal of PsbD was rate limiting but in our experiments under low to moderately high blue light the clearance of PsbD (Fig. 2b) equaled or exceeded the clearance of PsbA (Fig. 2a), at least within the short-term lincomycin incubations of 90 min that we applied.

We approached the estimation of \(k'_\text{psbA}\), the rate constant for removal of inactive PsbA from [PSII\textsubscript{inactive}] by estimating [PSII\textsubscript{inactive}] as [PsbA]\textsubscript{free} – [PSII\textsubscript{active}] using our immunoquantifications of [PsbA] and our parallel oxygen flash yield estimates of [PSII\textsubscript{active}] from the same subset of cultures. Then, we used the ratio [PSII\textsubscript{inactive}]/[PsbA] to estimate [PSII\textsubscript{inactive}] for those cultures for which volume limitations precluded direct measurement of [PSII\textsubscript{active}] using oxygen flash yields. We experimentally measured loss of PsbA from the [PsbA\textsubscript{total}] pool = [PSII\textsubscript{inactive}] + [PSII\textsubscript{activenpq}], in the presence of lincomycin to block the replacement of PsbA. We assume [PsbA\textsubscript{free}] ~ 0 as PsbA synthesis is coordinated with assembly. To infer \(k'_\text{psbA}\):
dPsbA/dt = (k_{PsbA} + \mu) \times \{[\text{PSII}_{\text{inactive}}] + [\text{PSII}_{\text{activepq}}]\} \\
= (k_{PsbA} + \mu) \times [\text{PsbA}_{\text{total}}]

The loss of PsaA actually occurs as

dPsbA/dt = (k'_{PsbA} + \mu) \times [\text{PSII}_{\text{inactive}}]

dPsbA/dt = (k'_{PsbA} + \mu) \times [\text{PSII}_{\text{inactive}}] \\
= (k_{PsbA} + \mu) \times [\text{PsbA}_{\text{total}}]

\langle k'_{PsbA} \rangle = \langle k_{PsbA} + \mu \rangle \times [\text{PsbA}_{\text{total}}]/[\text{PSII}_{\text{inactive}}] - \mu

And, as an estimate of the content of [PsbA_{activepq}] that does not rely upon the slow, high-volume oxygen flash yield method:

\[ [\text{PsbA}_{\text{activepq}}] \sim [\text{PsbA}_{\text{total}}] - [\text{PSII}_{\text{inactive}}] \]

algebraically, from rate equation 2, above, under steady state conditions:

\[ [\text{PsbA}_{\text{activepq}}] = (\text{fmol PsaA} \ \mu \text{protein}^{-1})/(1 + (k_{PsbA}/(k'_{PsbA} + \mu))) \]

As a cross check on our use of oxygen flash yields to estimate [PsbA_{activepq}], in Fig. S1 in Supplementary material, we compare the prediction of

\[ [\text{PsbA}_{\text{activepq}}] = (\text{fmol PsaA} \ \mu \text{protein}^{-1})/(1 + (k_{PsbA}/(k'_{PsbA} + \mu))) \]

with the measured [PsbA_{activepq}] from oxygen flash yields, showing good correspondence of the estimators. This cross-validation serves two purposes; it lends confidence to a key equation of the PSII repair cycle, and it gives us a validated approach to estimate the content of active PSII using the low-sample volume approaches of immunooquantitation and rate constants, rather than the high-sample volume oxygen flash yield approach.

3. [PSII_{diss}]: the pool(s) of disassembled PSII sub-complex(es), awaiting reassembly with replacement sub-units of PsaA, and possibly with PsaD.

\[ d[\text{PSII}_{\text{diss}}]/dt = \{[\text{PSII}_{\text{inactive}}] \times k_{\text{PsbA}}\} + \{[\text{PSII}_{\text{total}}] \times \mu\} - \{[\text{PSII}_{\text{diss}}] \times (k_{\text{ass}} + \mu)\} \]

\text{(RateEquation3)}

We make the simplifying assumption that all the sub-complexes resulting from disassembly of PSII are present in an equal stoichiometry and can thus be represented as a single pool, even though studies with model cyanobacteria (reviewed in Nixon et al. 2010; Komenda et al. 2012) show that multiple different sub-complexes are present. For the rate equation for [PSII_{diss}], we include a growth term for the pool of \{[\text{PSII}_{\text{total}}] \times \mu\}, as balanced growth requires [PSII_{total}] to accumulate at the same rate as overall cell specific growth, but biosynthetically the new PSII subunits will first enter the [PSII_{diss}] pool. On the other hand, growth dilution acts upon all three pools and so we include the loss term \{[\text{PSII}_{\text{diss}}] \times \mu\} to reflect the dilutional removal of [PSII_{diss}] by growth. In Thalassiosira pseudonana CCMP 1335, our data indicates that the pool of [PSII_{diss}] is small relative to the other pools, although Wu et al. (2011) found evidence that [PSII_{diss}] could be a significant pool in Thalassiosira pseudonana CCMP 1014. As PSII repair is cyclic, under steady state these three rate equations algebraically sum to zero, with no change in [PSII_{total}], when normalized to total cellular protein.

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