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Investigating the Photoprotective Role of Cytochrome *b*-559 in Photosystem II in a Mutant with Altered Ligation of the Haem

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Despite many years of study, the physiological role of cytochrome *b*-559 (Cyt *b*-559) within the photosystem II (PSII) complex still remains unclear. Here we describe the analysis of a mutant of the green alga *Chlamydomonas reinhardtii* in which the His ligand to the haem, provided by the alpha subunit, has been replaced by a Cys residue. The mutant is unable to grow photoautotrophically but can assemble oxygen-evolving PSII supercomplexes to 15–20% of the levels found in the wild-type control. Haem is still detected in the isolated PSII supercomplexes but at sub-stoichiometric levels consistent with weaker binding to the mutated cytochrome. Analysis of PSII activity in cells indicates slowed electron transfer in the mutant between plastoquinones Q_A and Q_B. We show that PSII activity in the mutant is more sensitive to chronic photoinhibition than the WT control because of two effects: a faster rate of damage and an impaired PSII repair cycle at the level of synthesis and/or incorporation of D1 into PSII. We also demonstrate that Cyt *b*-559 plays a role during the critical stage of assembling the Mn_4CaO_5 cluster. Overall we conclude that Cyt *b*-559 optimises electron transfer on the acceptor side of PSII and plays physiologically important roles in the assembly, repair and maintenance of the complex.

Keywords: Chloroplast mutant • Photoinhibition • PSII repair.

Abbreviations: Car_{D2}, carotenoid bound to the D2 subunit; Chl, chlorophyll; Cyt, cytochrome; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FeCN, potassium ferricyanide; LHCs, light-harvesting complexes; LHCII, light-harvesting chlorophyll-a/b-binding complexes of photosystem II; Mn_4CaO_5, manganese/calcium cluster catalysing water oxidation in PSII; PI, photoinhibition; PSII, photosystem II; PSII SC, photosystem II-LHCII supercomplex; Q_A, primary quinone electron acceptor in PSII; Q_B, secondary quinone electron acceptor in PSII; ROS, reactive oxygen species; S_2 state, oxidation state of the Mn_4CaO_5 cluster that has accumulated two oxidizing equivalents.

Introduction

The photosystem II (PSII) complex found in all oxygen-evolving photosynthetic organisms functions as a water-plastoquinone oxidoreductase, using light energy to catalyze water splitting and to initiate an electron transfer cascade that ultimately generates the ATP and NADPH required for photosynthesis (Diner and Babcock 1996). PSII is also a weak link in the photosynthetic electron transport chain and is prone to irreversible light-induced damage, which unless repaired leads to a net reduction in the rate of photosynthesis, sometimes termed photoinhibition (Vass 2012). A number of protective mechanisms exist to prevent damage to PSII (Takahashi and Badger 2011) and an elaborate PSII repair cycle operates once damage has occurred to replace damaged subunits, usually the D1 reaction center subunit, by a newly synthesized version (Nixon et al. 2010, Komenda et al. 2012).

Cytochrome *b*-559 (Cyt *b*-559) is a ubiquitous component of PSII located close to the D1 and D2 reaction center subunits (Nanba and Satoh 1987) and is composed of a heterodimer of the α (PsbE) and ß (PsbF) subunits with the haem molecule ligated by single histidine residues in each subunit. The crystal structures of cyanobacterial PSII have confirmed that Cyt *b*-559 is adjacent to the D2 subunit of the reaction center with the haem positioned towards the cytoplasmic side of the membrane approximately 25 Å from the binding site for the secondary quinone electron acceptor, Q_B, and 11.6 Å from a carotenoid, Car_{D2}, bound to the D2 subunit (Ferreira et al. 2004, Loll et al. 2005, Guskov et al. 2009, Umena et al. 2011).

Despite extensive research the physiological role of Cyt *b*-559 still remains unresolved, although important new insights

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are coming from analysis of mutants generated in the green alga *Chlamydomonas reinhardtii* (Morais et al. 2001, Ma et al. 2007), the cyanobacteria *Synechocystis* 6803 (Hung et al. 2007, Chiu et al. 2009, Hung et al. 2010, Chiu et al. 2013) and *Thermosynechococcus elongatus* (Guerrero et al. 2014) and the tobacco *Nicotiana tabacum* (Bondarava et al. 2003, Bondarava et al. 2010). Analysis of null mutants has revealed that Cyt *b*-559 plays a structural role during the early stages of PSII assembly, possibly acting as a nucleation factor (Morais et al. 1998, Komenda et al. 2004). Mutation of the His axial ligands of Cyt *b*-559 to Leu also has drastic effects on the accumulation of PSII (Pakrasi et al. 1991). PSII activity in those Cyt *b*-559 mutants that do accumulate PSII complexes is noticeably more sensitive to photoinhibition than WT in *C. reinhardtii* (Morais et al. 2001), *Synechocystis* 6803 (Hung et al. 2010) and tobacco (Bondarava et al. 2010), providing important experimental support for a physiological role for Cyt *b*-559 in photoprotection as suggested from earlier studies on isolated PSII complexes (reviewed by Shinopoulos and Brudvig 2012). However, the photoprotective mechanism still remains unclear.

Previously we showed that mutation of the His ligand to the haem in the PsbE subunit of *C. reinhardtii* resulted in mutants with reduced levels of functional PSII (Morais et al. 2001). PSII-LHCII supercomplexes could still be isolated from the Tyr and Met mutants with WT rates of oxygen-evolution, despite the lack of bound haem. These data therefore provided unambiguous evidence that the haem is not required for oxygen evolution by PSII. However, the possibility that the haem was lost during isolation of the PSII supercomplexes or had a role in modulating electron transfer could not be excluded. Here we describe the characterization of a new mutant, PsbE-H23C, expressing higher levels of PSII, with emphasis on the binding of haem, possible perturbation of electron transfer on the acceptor side of PSII, a role in efficient PSII repair and a role in assembly of the Mn_4CaO_5 oxygen-evolving cluster. Preliminary aspects of this work were presented at the 14th International Congress on Photosynthesis (Hamilton et al. 2008).

Results

Construction of His-tagged PsbE-H23C mutant

We have previously described the construction of *psbE* mutants in *C. reinhardtii* through transformation of the chloroplast genome (Morais et al. 2001). A similar experimental approach was used to make the PsbE-H23C mutant (as well as the WT control) except that a C-terminal His-tagged PsbH strain of *Chlamydomonas reinhardtii* (Cullen et al. 2007) was transformed to potentially allow isolation of His-tagged PSII. PCR analysis confirmed that the resulting transformed strains had segregated and contained the desired mutation (**Supplementary Fig. S1**). The strains were designated His-H23C and His-Pf3 (WT control).

His-H23C cells assemble PSII supercomplexes but are unable to grow photoautotrophically

The His-H23C mutant was unable to grow photoautotrophically and was therefore grown on medium supplemented with acetate. His-H23C gave oxygen-evolution rates of 15–40% of His-Pf3 consistent with the levels of D1 detected immunochemically in whole cell extracts (**Supplementary Fig. S2**). In line with a previous analysis of PsbE-H23Y and PsbE-H23M mutants (Morais et al. 2001), the His-H23C mutant assembled PSII-LHCII supercomplexes which could be isolated by sucrose density gradient centrifugation (**Fig. 1**). Analysis by SDS-PAGE and immunoblotting confirmed the presence of the PSII core proteins, various LHCII subunits and the PsbO, PsbP and PsbQ extrinsic proteins of PSII as previously reported (Morais et al. 2001) (**Fig. 1**). The oxygen-evolving activity of the PSII-LHCII supercomplexes was similar to that of His-Pf3 (approx. 450 μmol oxygen/mg Chl/h). Consistent with this, the amounts of photoreducible Q_A per Chl, which was determined from the initial amplitude of the flash-induced absorbance change at 320 nm at room temperature, were the same within the error limits (data not shown). In addition the light-minus-dark absorbance difference spectra recorded at 77 K revealed that the band shift centered at 543 nm induced by reduction of Q_A

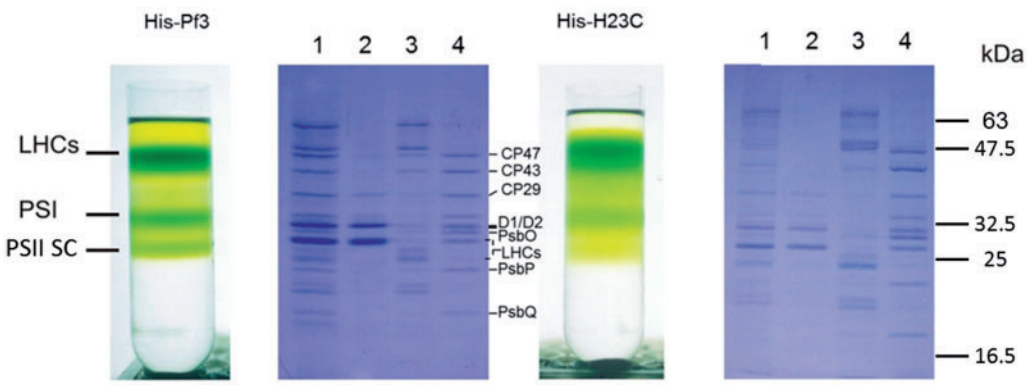

Fig. 1 Sucrose density gradients and SDS-PAGE analysis of detergent-solubilised His-Pf3 and His-H23C thylakoids. Lane 1, pure thylakoids; lane 2, LHC fraction; lane 3, PSI-LHCI supercomplex; lane 4, PSII-LHCII supercomplex. 5 μg Chl of thylakoids and 3 μg Chl of gradient material were loaded in each well. Protein bands were identified by immunoblotting according to Morais et al. (2001).

(the so-called 'C550' signal) was also about the same in both samples on an equal chlorophyll basis.

Fig. 2 shows the absorbance difference spectra of Cyt *b*-559 in the region of the α-band. The two sets of difference spectra were obtained by subtracting the absorbance spectrum of the sample with Cyt *b*-559 in the oxidised state (by treating with 2 mM potassium ferricyanide) from that with Cyt *b*-559 in the reduced state (by addition of 5–10 mM dithionite). The signal observed in isolated PS II supercomplexes from the mutant was only about 20% of that from the WT control. In addition, the band was slightly shifted to the red. The magnitude of this signal in the mutant was, however, variable and in some preparations could not be detected (**Supplementary Fig. S3**). Overall these data support the idea that haem can bind to the His-H23C mutant *in vivo* but is more easily lost during isolation of detergent-solubilized PSII.

PSII electron transfer is perturbed in His-H23C cells

Fig. 3 shows the chlorophyll fluorescence relaxation kinetics recorded for dark-adapted His-H23C and His-Pf3 cells following flash excitation. The relaxation of fluorescence yield reflects different re-oxidation routes of Q_A^- (Crofts 1993, Renger et al. 1995). The first fast phase reflects oxidation of Q_A^- by bound PQ and occurs on a few 100 microsecond time scale. The second fastest phase, which occurs on a few ms time scale, reflects re-oxidation of Q_A^- in centers that had an empty Q_B site prior to the flash. The third phase, operating on a seconds time scale reflects charge recombination between $Q_A^-Q_B/Q_AQ_B^-$ and the S_2 state of the Mn_4CaO_5 oxygen-evolving cluster.

Multi-component de-convolution of the measured fluorescence relaxation curves was calculated using a fitting function with two exponential components (corresponding to the fast and middle phases) and a hyperbolic component (corresponding to the slow phase) according to Vass et al. (1999) (see Materials and Methods). The first fast phase was 2.7 times faster in WT cells compared to His-H23C indicating that electron transfer from Q_A^- to bound plastoquinone was slowed in the mutant (**Table 1**). The slow middle phase was significantly faster in His-H23C cells compared to WT, 7.4 ± 8.6 ms compared to 47 ± 21 ms. This can be attributed to an increased availability of plastoquinone in mutant cells where PSII levels are depleted. The slow phase of fluorescence decay, which lasted several seconds in WT cells was much faster in His-H232C cells, 0.3 s compared to 2.17 s. DCMU was added to samples to determine if this faster charge recombination was due to an effect on the acceptor or donor side of PSII (**Fig. 3**). DCMU binds to the Q_B site of PSII and so charge recombination can only occur via Q_A^-/S_2. The rate of charge recombination between Q_A^- and S_2 was not affected in the presence of DCMU in mutant cells. Hence the faster slow phase in the absence of

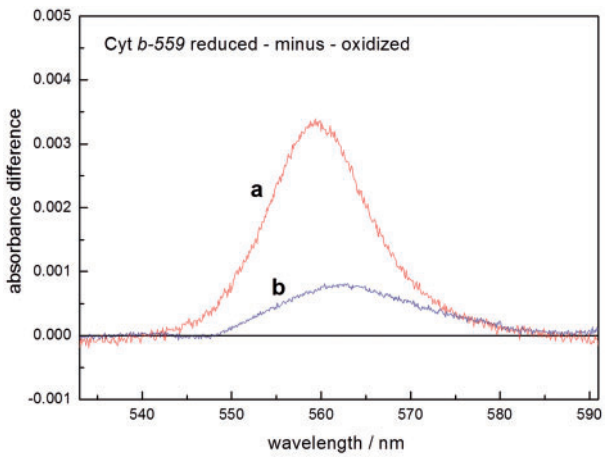

Fig. 2 Reduced – minus – oxidized absorbance difference spectra of Cyt *b*-559 in PS II supercomplexes from (a) His-Pf3 (WT control) and (b) mutant His-H23C. Cyt *b*-559 was reduced by the addition of 5–10 mM dithionite and oxidised by the addition of 2 mM potassium ferricyanide. The spectra have been normalized to a Chl concentration of A = 1 at the maximum in the Q_y region.

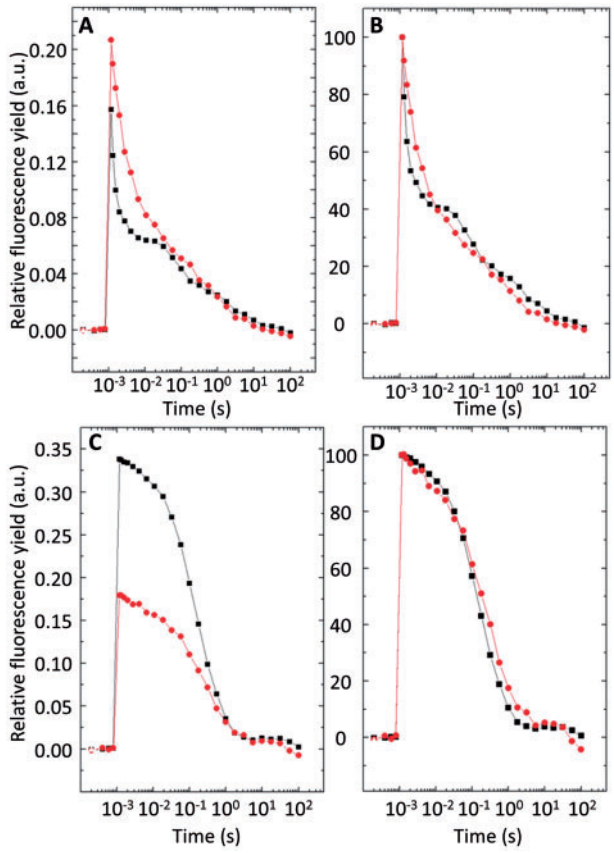

Fig. 3 Flash fluorescence measurements of His-Pf3 and His-H23C cells. Cells in the early log phase at 10 μg chlorophyll/ml were dark-adapted for 5 min before being subjected to a single saturating light flash in the presence and absence of DCMU. Chlorophyll fluorescence relaxation was detected using a double modulation fluorimeter. (A) Whole cells. (B) Whole cells with signal normalised to His-Pf3. (C) Whole cells plus DCMU. (D) Whole cells plus DCMU signal normalised to His-Pf3. Black squares His-Pf3. Red circles, His-H23C.

DCMU in the mutant indicates a shift to the left in the $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ equilibrium (Vass et al. 1999).

Effect of high light on PSII activity in WT and His-H23C cells

To assess whether PSII activity was more prone to damage in His-H23C, whole cells were subjected to high irradiance (1000 μmol photons.m^{-2}s^{-1}) and PSII oxygen-evolving activity was assessed by measuring light-saturated oxygen evolution in the presence of the artificial electron acceptors 2,6-dichloro-*p*-benzoquinone (DCBQ) and potassium ferricyanide (FeCN) (**Fig. 4A**). Cell plating assays confirmed that the His-H23C cells and WT control remained fully viable during the high light treatment (data not shown). After an initial decrease in oxygen-evolving ability, PSII activity in the WT control cells recovered to around 80% of their initial oxygen-evolving activity within 60 min. The initial drop and subsequent recovery in PSII activity was mirrored by changes in the abundance of PSII subunits in WT cells as determined by immunoblotting (**Fig. 4B**). By contrast, His-H23C cells showed a decrease in both PSII activity (**Fig. 4A**) and the levels of PSII subunits (**Fig. 4C**) over the high-light period.

To disentangle the effects of PSII repair from PSII damage, cells were exposed to high light in the presence of the protein synthesis inhibitor lincomycin (**Fig. 4**). Both WT and mutant cells displayed a rapid decrease in PSII activity, with loss of activity more pronounced in His-H23C (**Fig. 4A**). After 60 min no PSII activity was detected in His-H23C cells compared to around 20% in WT cells. Immunoblotting confirmed a decline in the levels of PSII subunits in both His-Pf3 and His-H23C consistent with their degradation (**Fig. 4B, C**).

Pulse-labeling experiments conducted in the light in the absence of lincomycin revealed that D1 synthesis was reduced in His-H23C compared to the WT control with levels of radiolabeled D1 declining rather than increasing during exposure of cells to light (**Fig. 5**).

A role for Cyt *b*-559 in assembly of the Mn_4CaO_5 cluster

Current models based on the redox properties of Cyt *b*-559 and the kinetics of its reduction and oxidation measured *in vitro* suggest that it might function to cycle electrons around PSII and play a possible physiological role in reducing oxidizing equivalents in PSII that accumulate under conditions of donor-side photoinhibition (PI) (reviewed by Shinopoulos and Brudvig 2012). One physiologically relevant situation where donor-side PI might be a major problem is during the light-driven assembly of the Mn_4CaO_5 oxygen-evolving complex (termed photoactivation) by PSII core complexes. To test this idea, we exploited the experimental procedure developed by Callahan and Chenaie (1985) in which *Chlamydomonas* cells are treated with hydroxylamine to reduce and inactivate the Mn_4CaO_5 cluster, the hydroxylamine is removed by washing, lincomycin is added to prevent protein synthesis and assembly of the Mn_4CaO_5 cluster in pre-assembled PSII complexes is followed in cells by measuring oxygen evolution as a function of time of exposure to light. In our experiments, three different light intensities (40, 400 and 1200 μmol photons.m^{-2}s^{-1}) were used over a period of 25 min (**Fig. 6**).

A striking observation from these experiments was the rapid photoactivation of PSII activity in the His-H23C cells exposed to 40 μmol μmol photons.m^{-2}s^{-1} followed by slow photoinactivation (**Fig. 6B**). By contrast, WT control cells showed a much slower rate of photoactivation but activity was more resistant to photoinactivation (**Fig. 6A**). Increasing the light intensity to 1200 μmol photons.m^{-2}s^{-1} increased the rate of photoactivation in WT control cells at the expense of some loss of activity. In the case of His-H23C, there was a decrease in the maximum amount of active PSII centers that could be activated at the higher irradiance (**Fig. 6B**). Overall, the yield of oxygen-evolving PSII centers after 5 min illumination, which reflects the efficiency of both photoactivation and photoprotection, decreased in the His-H23C mutant with increasing light intensity, whereas it increased for the WT control (**Fig. 6C**).

Discussion

We have investigated the possible physiological role of Cyt *b*-559 in a mutant in which one of the two axial ligands to the haem (PsbE-H23) has been replaced by Cys, which can potentially act as a ligand to the haem to allow assembly of the cytochrome and accumulation of PSII complexes. Like the earlier Met and Tyr mutants constructed at this position (Morais et al. 2001), oxygen-evolving PSII supercomplexes could be isolated from the Cys mutant, although at a reduced level compared to the WT control (**Fig. 1**). However, unlike the

Table 1 Decay kinetics of flash-induced variable fluorescence in His-Pf3 and His-H23C cells

Sample	Fast phase		Middle phase		Slow phase	
	$t_{1/2}$(μs)	[amp (%)]	$t_{1/2}$ (ms)	[amp (%)]	$t_{1/2}$ (s)	[amp (%)]
His-Pf3	243 ± 21	[63 ± 3]	47 ± 21	[34 ± 2.5]	2.17 ± 0.5	[2.5 ± 5]
His-Pf3 + DCMU	–	–	0.8 ± 1	[2.3 ± 1.5]	0.13 ± 0	[97 ± 1]
His-H23C	652 ± 104	[29 ± 2]	7.4 ± 8.6	[23 ± 4.5]	0.26 ± 0.04	[47 ± 1.4]
His-H23C + DCMU	–	–	1.5 ± 0.7	[8 ± 1]	0.13 ± 0	[92 ± 1]

Curves were analysed in terms of two exponential components (fast and middle phases) and one hyperbolic component (slow phase). Values in parentheses are relative amplitudes as a percentage of total variable fluorescence following the flash of light. Calculated according to Vass et al. (1999).

previous mutants, some residual haem could be detected in the complexes isolated from the His-H23C mutant. The lack of haem in some preparations indicates that the haem is weakly bound and can be lost during purification of PSII supercomplexes (**Supplementary Fig. S3**). This same possibility might also apply to the Met and Tyr mutants studied previously (Morais et al. 2001). In the case of *Synechocystis* 6803, the His ligand mutant H22Kα of PsbE also shows similar partial retention of haem in isolated PSII complexes (Hung et al. 2007). Interestingly the equivalent mutant to His-H23C in *Synechocystis* 6803 is much more perturbed showing only 4% of WT levels of oxygen evolution and 15 ± 8 % of PSII content (Hung et al. 2007), which might reflect differences in growth conditions causing different degrees of chronic photoinhibition.

Electron transfer in PSII is perturbed on the acceptor side

One defect that we detected in the His-H23C mutant from chlorophyll fluorescence measurements was a perturbation in electron transfer on the acceptor side of PSII (**Fig. 3**). Forward electron transfer is slowed and charge recombination is accelerated (**Table 1**). Measurements in the presence of DCMU demonstrate that this perturbation is likely due to a destabilisation of Q_B^-, indicating a role for Cyt *b*-559 in optimizing electron transfer between Q_A and Q_B, through modulation of the free energy gap between the quinones. This effect on Q_B/Q_B^- might be due to indirect structural effects as the herbicide DCMU, which binds close to the Q_B-binding pocket in PSII, shows tighter binding in His-H23C cells than in WT (**Supplementary Fig. S4**).

Cyt *b*-559 protects PSII under high light conditions

We found that oxygen-evolving PSII complexes in His-H23C are more susceptible to PI under high light conditions than in WT control cells (**Figs. 4A**, **6**). The rate of damage to PSII in the mutant is clearly greater than that seen for the WT control, in line with observations made on the Tyr and Met mutants (Morais et al. 2001). This might be due to increased production of damaging species in the mutant or perturbed photoprotection. Possible mechanisms for this protective effect include a role as a superoxide dismutase helping to remove damaging

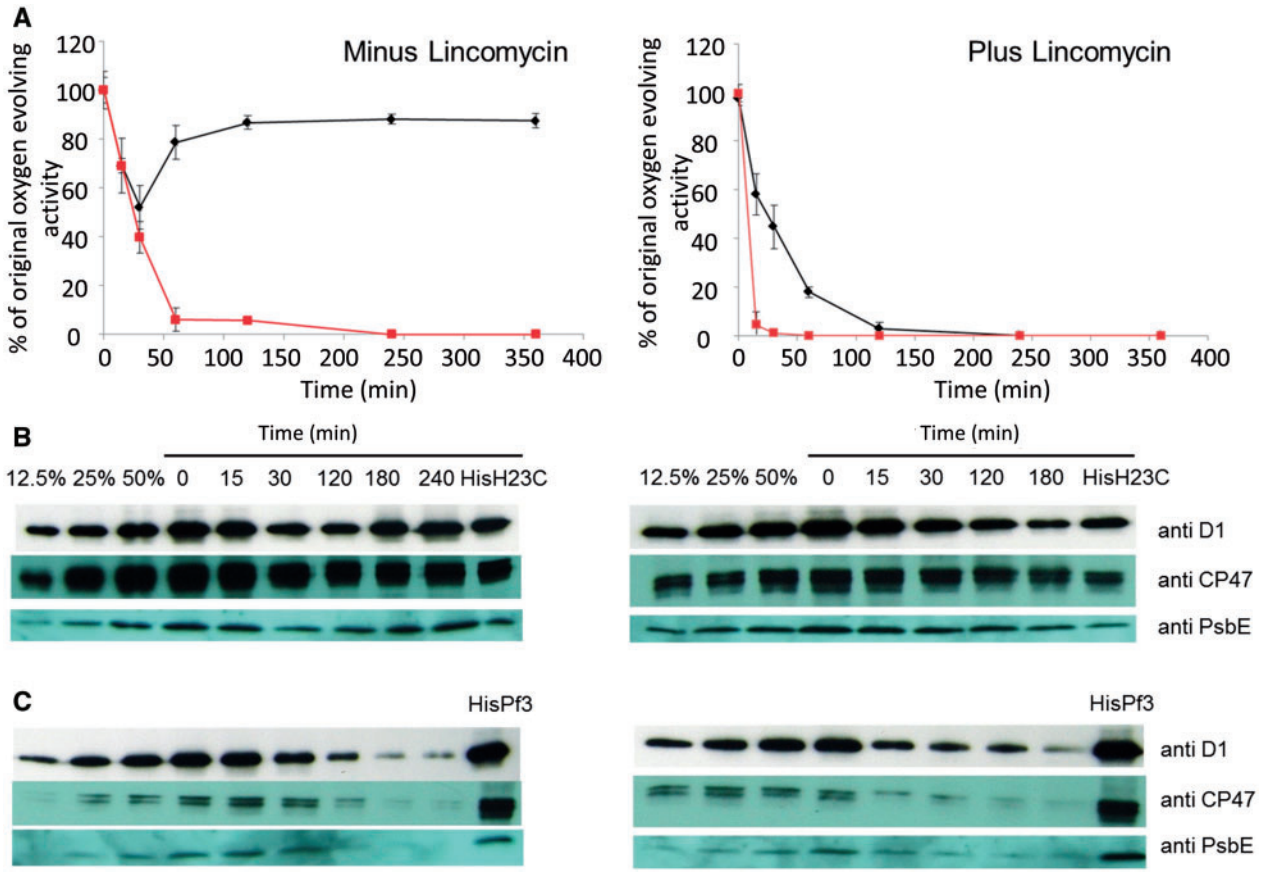

Fig. 4 Response of His-Pf3 and His-H23C cells to high light. Cells were exposed to high light (1000 μmol photons.m^{-2} s^{-1}) in the presence and absence of lincomycin (150 μg/ml). (A) The percentage of oxygen evolution is plotted against time. Black diamonds, His-Pf3; Red squares, His-H23C. Oxygen evolution was measured in the presence of DCBQ and $K_3Fe(CN)_6$. Initially His-Pf3 cells evolved 165 ± 2 μmol oxygen/mg Chl/h and His-H23C cells 47 ± 3 μmol oxygen/mg Chl/h. Immunoblotting using antibodies raised against PSII subunits was used to determine protein levels in His-Pf3 (B) and His-H23C (C) cells. With the exception of the dilution series, 1 μg Chl was loaded.

reactive oxygen species (Ananyev et al. 1995), a role in regulating the levels of chlorophyll cations in PSII that quench excitation (reviewed by Shinopoulos and Brudvig 2012) and a role as an emergency electron donor in its reduced state to help recycle electrons to the donor side of PSII via Car_{D2}, a β-Car molecule bound to the D2 subunit (Umena et al. 2011). Car_{D2} can donate electrons to $P680^+$ at a low rate (Shinopoulos et al. 2014) with reduction of $P680^+$ correlated with oxidation of Cyt *b*-559 (Buser et al. 1992, Magnuson et al. 1999). This pathway would only be significant when electron donation from the Mn_4CaO_5 cluster is absent, such as during photoactivation, and when PSII is vulnerable to so-called donor-side PI (Vass 2012). In this model Cyt *b*-559 could provide the electrons to re-reduce Car_{D2} and in turn would be reduced by reduced plastoquinone occupying the Q_B site or the Q_C site identified by Kaminskaya et al. (2007).

Car_{D2} also protects PSII by physically quenching singlet oxygen generated within the reaction centre (reviewed by Telfer 2014). More recently, it has been proposed that chemical quenching of singlet oxygen by carotenoid in PSII to form endoperoxides and, ultimately, various cleavage products might play an important signaling role to indicate high light/photooxidative stress in the cell (reviewed by Ramel et al. 2013). Given this possibility, Cyt *b*-559 might function to protect Car_{D2} from damage and indirectly to regulate acclimation to high light stress.

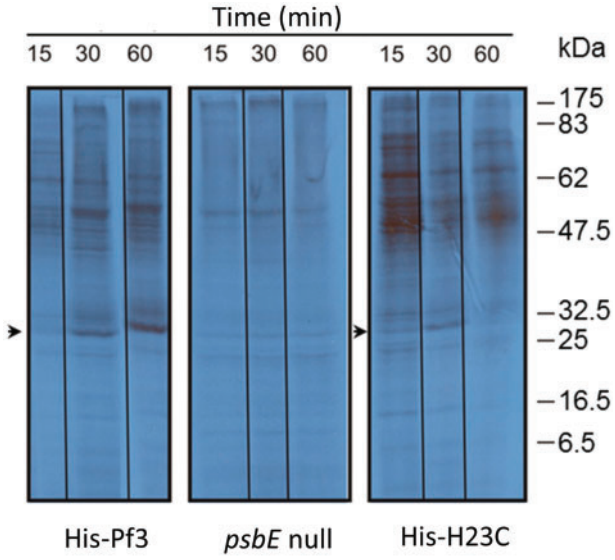

Fig. 5 Effect of strong light on synthesis of chloroplast-encoded proteins in His-Pf3, *psbE* null and His-H23C cells. Cells were incubated at 1000 μmol photons.m^{-2} s^{-1} for 30 min and subsequently incubated in the dark in the presence of cycloheximide (100 μg/ml) for 5 min. Pulse labelling of chloroplast-encoded proteins was performed with [^{14}C]-sodium acetate (5 μCi/ml) for 30 min following exposure to 40 μmol photons.m^{-2}.s^{-1} for 15, 30 and 60 min. Cells were solubilised and separated by SDS PAGE. 2 μg Chl was loaded in each lane. D1 protein is indicated by arrows.

Cyt *b*-559 has also been suggested to function as a plastoquinol oxidase (Bondarava et al. 2003, Bondarava et al. 2010). However, we detected no defect in plastoquinol oxidation in the dark in the His-H23C strain (data not shown).

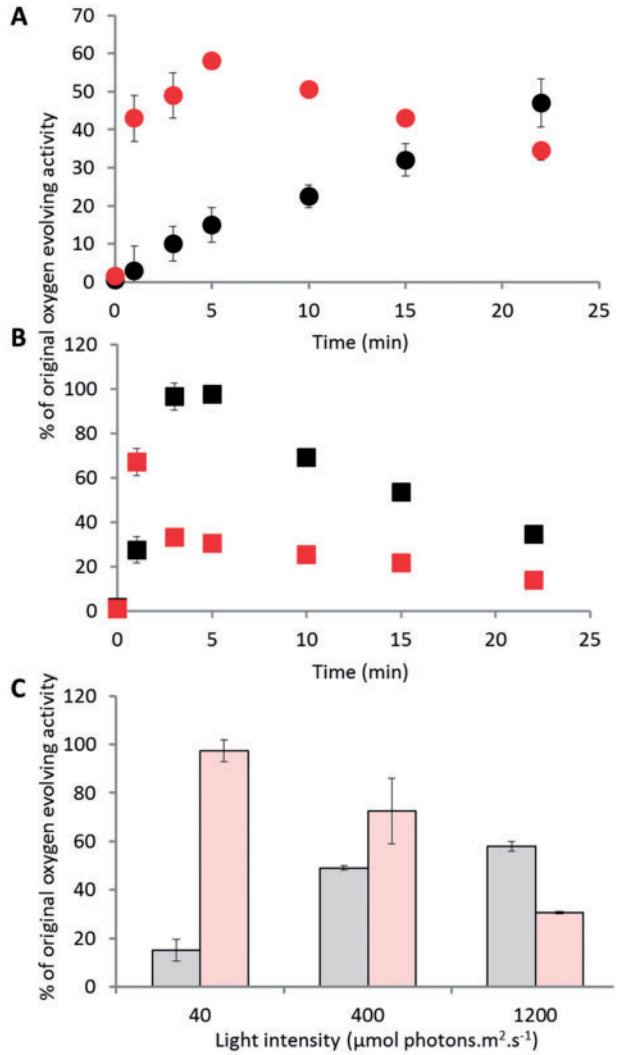

Fig. 6 Photoactivation measurements in whole cells. His-H23C and His-Pf3 cells were treated with hydroxylamine and washed thoroughly. Following dark incubation, cells were exposed to light intensities of 40, 400 and 1200 μmol photons.m^{-2}.s^{-1} Oxygen evolution was measured at saturating light in the presence of 1 mM $K_3Fe(CN)_6$ and 1 mM 2,6-dichloro-*p*-benzoquinone (DCBQ). Photoactivation of His-Pf3 (circles, panel A) and His-H23C cells (squares, panel B) at 40 (black symbols) and 1200 μmol photons.$m^{-2}s^{-1}$ (red symbols). Data are the result of three biological replicates with error bars indicating standard deviation; error bars not shown are less than the size of the symbol. (C) Oxygen-evolving activity of His-Pf3 and His-H23C cells after five minutes exposure at different light intensities. Grey bars, His-Pf3; pale red bars, His-H23C. Data are the result of three biological replicates. Initial rates of oxygen evolution were 125 ± 5 μmol O_2/mg Chl/h for His-Pf3 and 42 ± 2 μmol O_2/mg Chl/h for His-H23C cells.

A role for Cyt *b*-559 in photoactivation

One striking feature of the photoactivation data in Fig. 6 was the more rapid assembly of the Mn_4CaO_5 cluster in the mutant compared to the WT control at low light intensities. The reason for this is unclear, but we speculate that re-reduction of $P680^+$ via cyclic electron flow around PSII via Cyt *b*-559 and Car_{D2} might compete with the photoactivation process. When this pathway is less active, such as possibly in the His-H23C mutant, photoactivation might be more effective but at the expense of reduced capacity to photoprotect the PSII holoenzyme at higher irradiances. In addition there was a noticeable decline in the levels of oxygen-evolving PSII that could be assembled at early time points in His-H23C at higher irradiances (Fig. 6C), which hints towards a photoprotective role in PSII complexes lacking the assembled cluster not just oxygen-evolving complexes. However, further work is required to establish this fact.

Cyt *b*-559 is important for the PSII repair cycle

Irreversibly photodamaged PSII complexes can be reactivated through the operation of the so-called PSII repair cycle in which damaged complexes undergo partial disassembly, the damaged subunit (usually the D1 subunit) is removed through proteolysis, a newly synthesized subunit is inserted into the disassembled PSII complex and the PSII complex is reactivated through reattachment of undamaged subunits and light-driven assembly of the Mn_4CaO_5 oxygen-evolving cluster (reviewed by Nixon et al. 2010, Komenda et al. 2012). Besides being more prone to PSII damage, the His-H23C mutant is also much less effective at repairing damaged PSII centers. This is clearly shown in the classic PSII repair assay in Fig. 4 in which PSII activity is measured either in the absence of lincomycin (to permit operation of the PSII repair cycle) or in the presence of lincomycin (to block the cycle at the level of protein synthesis). The His-H23C mutant is still able to degrade PSII subunits during light stress but from Fig. 5 is impaired in the synthesis of PSII proteins and/or insertion into PSII. Cyt *b*-559 is known to be required for assembly of *de novo* PSII in *C. reinhardtii* (Morais et al. 1998). In the case of *Synechocystis* 6803, Cyt *b*-559 forms an assembly complex with the D2 subunit which then attaches to a D1/PsbI sub-complex to form a PSII reaction center assembly complex (Komenda et al. 2004, Knoppová et al. 2014). Replacement of damaged D1 during PSII repair is, however, thought to occur at the level of the RC47 complex (a PSII core complex lacking CP43) (Boehm et al. 2012, Komenda et al. 2012). Given the proximity of Cyt *b*-559 to the Q_B site in PSII, our data raise the possibility that mutation of Cyt *b*-559 in the His-H23C mutant might be compromising insertion of newly synthesized D1 into the RC47 complex. A marked phenotype of the His-H23C mutant is the reduced ability to synthesize D1 upon exposure to high light (Fig. 5). Possible reasons include loss of RC47 complexes from the membrane preventing co-translational D1 replacement (Zhang et al. 2001) and inhibition of D1 translation due to oxidative stress (Nishiyama et al. 2006).

Conclusions

The data presented here support multiple roles for Cyt *b*-559 in photoprotection. During high light treatment, PSII in the His-H23C mutant is more susceptible to photodamage in the absence of protein synthesis. The mutant cells are also less able to acclimate to high-light conditions and repair PSII. In addition, Cyt *b*-559 helps stabilize Q_B^-, which might improve the efficiency of PSII and help prevent charge recombination events that can lead to the generation of ROS and inactivation of PSII (Vass 2012). Evidence for a role in photoactivation is also presented.

Materials and methods

Strains and growth conditions

The C-terminal His-tagged PsbH strain of *C. reinhardtii* strain, H-his (Cullen et al. 2007), and the *psbA* null strain, FuD7 (Bennoun et al. 1986), were obtained from Dr. S. Purton (UCL, UK). Cells were grown in Tris-acetate phosphate (TAP) medium or high salt medium (Harris 1989) as previously described (Morais et al. 1998). Cell viability was performed by plating a dilution series of cell cultures onto TAP plates and determining the colony forming units per ml.

Transformation of *C. reinhardtii*

Plasmid DNA containing the *aadA* selectable marker (Goldschmidt-Clermont 1991) conferring resistance to spectinomycin and streptomycin and a point mutation at H23 was obtained from Dr. F Morais (Imperial College London, UK). Plasmid DNA was precipitated onto gold particles (0.6 μm diameter) and introduced into the chloroplast genome of psbH His cells using a biolistic method employing a Bio-Rad Biolistic PDS1000/He system (Boynton and Gillham 1993). Transformants were selected on spectinomycin plates and homoplasticity was confirmed by PCR analysis according to Morais et al. (2001).

Assay of Cyt *b*-559

Absorbance difference spectra of Cyt *b*-559 at their α-band were measured in a Cary 1E UV/VIS-spectrophotometer (Varian). Spectra were recorded with data intervals of 0.1 nm and a spectral bandwidth of 1 nm. The difference spectra were obtained by subtracting the absorbance spectrum of the sample with Cyt *b*-559 in the oxidized state from that with Cyt *b*-559 in the reduced state. Cyt *b*-559 was reduced by the addition of 5–10 mM dithionite and oxidized by the addition of 2 mM potassium ferricyanide.

Isolation of PSII-LHCII supercomplexes

Thylakoid membranes were solubilized by 25 mM n-dodecyl-ß-D-maltoside and fractionated on sucrose-density gradients as described by Morais et al. (2001). The lowest band in the gradient corresponded to the PSII-LHCII supercomplexes.

SDS PAGE and immunoblotting

The protein composition of thylakoids was analysed on 12% (w/v) polyacrylamide gels containing 0.7 M urea using the Laemmli buffer system (Laemmli 1970). Immunoblotting was carried out according to Dunn (1986) as described by Morais et al. (2001). The antibody used to detect PsbE was generated by Morais et al. (1998). The D1 antibody is specific for the N-terminal fragment of spinach, a gift from Roberto Barbato (Barbato et al. 1991). The CP47 antibody was also a gift from R. Barbato.

Chlorophyll fluorescence measurements

Flash-induced increase and subsequent decay of chlorophyll fluorescence yield was measured using a double-modulation fluorometer (FL-3000, Photon System Instruments, Brno) as described by Trtilek et al. (1997). For measurements cells were harvested in their late exponential growth phase. After centrifugation (4000 × g for 10 min) cells were resuspended in fresh culture medium to achieve a final Chl concentration of 5 $\mu g.mL^{-1}$. The instrument's LED system provides both single turnover saturating actinic flashes (20 µs, 639 nm) and measuring flashes (8 µs, 620 nm). Fluorescence decay was recorded in the 150 µs to 100 s time range on a logarithmic time scale. Flash-induced fluorescence relaxation curves were analyzed as described by Vass et al. (1999). Multicomponent deconvolution of the monotonically decreasing curves was done by using a fitting function with two exponential components (fast and middle phase) and one hyperbolic component (slow phase): $F_{v,corr} = A_1\exp(-t/T_1) + A_2\exp(-t/T_2) + A_3/(1 + t/T_3) + A_0$ where $F_v = F(t) - Fo$, $F(t)$ is the fluorescence yield at time t, Fo is the basic fluorescence level before the flash, A_1–A_3 are the amplitudes, and T_1–T_3 are the time constants. Non-decaying fluorescence component in the time span of the measurement is described by a constant A_0 amplitude.

Photoinhibition and oxygen evolution

The effect of photoinhibition was measured according to Morais et al. (2001). Cells were grown in TAP medium at 100 µmol photons.$m^{-2}s^{-1}$ and collected in the exponential phase of growth at a Chl concentration of 10 µg/ml, placed in flat glass dishes at 25°C, stirred, and subjected to heat-filtered high-light irradiation of 1000 µmol photons.$m^{-2}s^{-1}$, provided by an apparatus equipped with a 1-kilowatt tungsten-halogen lamp. The rate of damage to PSII was determined by adding lincomycin to 10 µM. PSII activity was assessed by determining the light-saturated oxygen evolution in the presence of 1 mM $K_3Fe(CN)_6$ (FeCN) and 1 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) using a Clark electrode (Hansatech Instruments Ltd., Norfolk, UK) as described by Morais et al. (2001). DCBQ is cell permeable whereas FeCN is impermeable but able to oxidize reduced DCBQ.

Pulse-labeling

Pulse-labeling was carried out according to van Wijk et al. (1994) with modifications. Cells in the early-mid log phase were suspended in HSM medium to a Chl concentration of 10 µg/ml and incubated under low light (40 µmol photons.$m^{-2}s^{-1}$) for 2 h and exposed to strong light (1000 µmol photons.$m^{-2}s^{-1}$) for 30 min. Cells were then incubated in the presence of cycloheximide (100 µg/ml) for 5 min in the dark. Pulse labeling of chloroplast-encoded proteins was performed with [^{14}C]-sodium acetate (5 µCi/ml) for 30 min following exposure to 40 µmol photons.$m^{-2}s^{-1}$ for 15, 30 and 60 min immediately after incubation with cycloheximide. Total cell proteins were solubilized and separated by SDS-PAGE and radiolabeled proteins detected by phosphorimaging.

Photoactivation

Photoactivation of PSII was measured in whole cells according to Callahan and Cheniae (1985). Cells were grown in liquid culture to the mid-exponential phase under constant actinic light at 100 µmol photons.$m^{-2}s^{-1}$. Cells were collected and suspended in TAP medium to a Chl concentration of 1 mg/ml. Cells at a Chl concentration of 200 µg/ml were extracted with 10 mM NH_2OH (which is cell permeable) for 1 min in darkness to remove the Mn cluster and then diluted 27-fold with 20 mM Na-phosphate buffer (pH 6.0) before collection by centrifugation, and washing again. Cells were re-suspended in TAP to a Chl concentration of 1 mg/ml and lincomycin added to 10 µM. Cells were then exposed to a range of light intensities at 20°C and oxygen evolution measured in the presence of 1 mM FeCN and 1 mM DCBQ.

Supplementary data

Supplementary data are available at PCP online.

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