

First induced plastid genome mutations in an alga with secondary plastids: psbA mutations in the diatom Phaeodactylum tricornutum (Bacillariophyceae) reveal consequences on the regulation of photosynthesis

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First induced plastid genome mutations in an alga with secondary plastids: psbA mutations in the diatom Phaeodactylum tricornutum (Bacillariophyceae) reveal consequences on the regulation of photosynthesis

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1	FIRST INDUCED PLASTID GENOME MUTATIONS IN AN ALGA WITH
2	SECONDARY PLASTIDS: PSBA MUTATIONS IN THE DIATOM
3	PHAEODACTYLUM TRICORNUTUM (BACILLARIOPHYCEAE) REVEAL
4	CONSEQUENCES ON THE REGULATION OF PHOTOSYNTHESIS ¹
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24 Abstract

25 Diatoms are unicellular eukaryotes playing a crucial role in the biochemistry and ecology 26 of most aquatic ecosystems, especially because of their high photosynthetic productivity. 27 They often have to cope with a fluctuating light climate and a punctuated exposure to 28 excess light, which can be harmful for photosynthesis. To gain insight into the regulation 29 of photosynthesis in diatoms, we generated and studied mutants of the diatom 30 *Phaeodactylum tricornutum* carrying functionally altered versions of the plastidic *psbA* 31 gene encoding the D1 protein of the photosystem II (PSII) reaction center. All analyzed 32 mutants feature an amino acid substitution in the vicinity of the Q_B binding pocket of D1. 33 We characterized the photosynthetic capacity of the mutants in comparison to wild-type 34 cells, focusing on the way they regulate their photochemistry as a function of light 35 intensity. The results show that the mutations resulted in constitutive changes of PSII 36 electron transport rates. The extent of the impairment varies between mutants depending 37 on the proximity of the mutation to the Q_B binding pocket and/or to the non-heme iron 38 within the PSII reaction center. The effects of the mutations described here for P. 39 tricornutum are similar to effects in cyanobacteria and green microalgae, emphasizing the 40 conservation of the D1 protein structure among photosynthetic organisms of different 41 evolutionary origins. 42

Key words: diatom, D1 protein, electron transport, chlorophyll fluorescence, herbicide,
photosystem II, Q_B pocket

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- 46 Abbreviations: Chl a and Chl c, chlorophyll a and c; DCMU, (3-(3, 4-diclorophenyl)-1,1-
- 47 dimethylurea), LHC, light-harvesting complex; OEC, oxygen evolving complex; PAM,
- pulse amplitude modulation; PQ, plastoquinone; PSI and PSII, photosystem I and II; QA 48
- 49 and Q_B, quinone A and B; PSII RC, PSII reaction center; WT, wild-type.

л. . A and B,

50 Introduction

51 Diatoms (Heterokontophyta, Bacillariophyceae) are a major group of microalgae 52 ubiquitous in all marine and freshwater ecosystems. With probably more than 10.000 53 species, their biodiversity is among the largest of photosynthetic organisms, just after the 54 higher plants (Mann 1999). Diatoms are assumed to contribute to about 40 % of the 55 aquatic primary production (i.e. 20% of the annual global production), and to play a 56 central role in the biochemical cycles of silica (which is part of their cell-wall) and 57 nitrogen (Sarthou et al. 2005). Their productivity has contributed largely to the structure 58 of contemporary aquatic ecosystems (Falkowski et al. 2004). In contrast to the supposed 59 primary origin of red algae, green algae and higher plants, diatoms originate from a 60 secondary endosymbiotic event in which a non-photosynthetic eukaryote probably 61 engulfed a eukaryotic photosynthetic cell related to red algae and transformed it into a plastid (Keeling 2004). This peculiar evolution has led to complex cellular functions and 62 63 metabolic regulations recently highlighted by the publication of the genome of two 64 diatom species (Armbrust et al. 2004, Bowler et al. 2008), Thalassiosira pseudonana and *Phaeodactylum tricornutum.* It includes aspects of photosynthesis (Wilhelm et al. 2006), 65 photoacclimation (Lavaud 2007), carbon and nitrogen metabolisms (Allen et al. 2006, 66 67 Kroth et al. 2008), and response to nutrient starvation (Allen et al. 2008). 68 As for most microalgae, the photosynthetic efficiency and productivity of diatoms 69 strongly depend on the underwater light climate (MacIntyre et al. 2000). Planktonic as 70 well as benthic diatoms tend to dominate ecosystems characterised by highly turbulent 71 water bodies (coasts and estuaries) where they have to cope with an underwater light

72 climate with high frequency irradiance fluctuations coupled with large amplitudes.

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73	Depending on the rate of water mixing, diatoms can be exposed to punctual or chronic
74	excess light, possibly generating stressful conditions which impair photosynthesis (i.e.
75	photo-inactivation/-inhibition) (Long et al. 1994, Lavaud 2007). In higher plants and
76	cyanobacteria, the processes of PSII reaction center photo-inactivation/-inhibition are
77	strongly influenced by the redox state of the acceptor side of photosystem II (PS II) with
78	<u>quinones (Q_A and Q_B)</u> as primary electron acceptor (Vass et al. 1992, Fufezan et al.
79	2007).
80	Here we report the generation and characterization of four <i>psbA</i> mutants of <i>P</i> .
81	tricornutum. All mutants feature distinct amino acid exchanges in the D1 protein of PSII
82	close to or within the Q_B binding pocket. The point mutations resulted in a constitutive
83	impairment of the PSII electron transfer in all mutants to different extents. To our
84	knowledge this is the first report on similar mutants in an alga with secondary plastids.
85	
86	Material and Methods
87	Strains and media used for producing the psbA mutants of Phaeodactylum tricornutum
88	Phaeodactylum tricornutum Bohlin (University of Texas Culture Collection,
89	strain 646) was grown at 22°C under continuous illumination at 50 μ mol photons m ⁻² s ⁻¹
90	in Provasoli's enriched F/2 seawater medium (Guillard and Ryther 1962) using 'Tropic
91	Marin' artificial seawater at a final concentration of 50%, compared to natural seawater.
92	When used, solid media contained 1.2% Bacto Agar (Difco, Sparks, USA).
93	
94	Generation of psbA mutants from P. tricornutum

95	Construction of plasmid transformation vectors - Four transformation vectors were
96	constructed harboring a 795-bp $psbA$ fragment containing the Q_B binding pocket. The
97	psbA inserts of each vector carried individual point mutations leading to different
98	substitutions of the amino acid Serine encoded by the PsbA (D1) codon 264 (for details
99	and a vector map see Supplementary Fig. S1). In addition to the non-synonymous point
100	mutations in codon 264, a second, silent point mutation was introduced into codon 268
101	(TCT to TCA, nt position 804) without changing the encoded amino acid. The purpose of
102	the second point mutation was to delete a BssSI restriction site, thus allowing easy RFLP
103	screening of putative transformants.
104	Biolistic transformation of P. tricornutum - Transformation of P. tricornutum was
105	performed using a Bio Rad Biolistic PDS-1000/He Particle Delivery System (Bio-Rad,
106	Hercules, USA) as described previously (Kroth, 2007). Gold Particles with a diameter of
107	0.1 µm served as microcarriers for the DNA constructs. Bombarded cells were allowed to
108	recover for 24 h before being suspended in 1 mL of sterile F/2 50% medium.
109	Transformants (250 μ L) were selected at 21°C under constant illumination (35 μ mol
110	photons m ⁻² s ⁻¹) on agar plates containing 5 10 ⁻⁶ M DCMU <u>herbicide (3-(3, 4-</u>
111	diclorophenyl)-1,1-dimethylurea) and repeatedly streaked on fresh solid selective
112	medium to obtain full segregation of the mutation.
113	
114	Isolation of DNA and sequencing of wild-type and mutant psbA genes

115 Total nucleic acids from the WT and mutant cells were isolated via a

116 cetyltrimethylammoniumbromide (CTAB)-based method (Doyle and Doyle 1990). Prior

117 to the mutagenesis of *P. tricornutum* the WT *psbA* gene and the surrounding genes were

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118	sequenced (NCBI accession no AY864816) via primer walking (GATC, Konstanz,
119	Germany). For the molecular characterization of mutants, a 795 bp fragment of the <i>psbA</i>
120	gene was amplified as described in Fig. S1 and both strands were fully sequenced
121	(GATC, Konstanz, Germany).
122	
123	Cell cultivation and preparation for physiological measurements
124	P. tricornutum WT and mutant cells were grown in 200 mL sterile F/2 50% medium
125	(Guillard and Ryther 1962) at 21°C in airlift columns continuously flushed with sterile air.
126	The cultures were illuminated at a light intensity of 50 μ mol photons m ⁻² s ⁻¹ with white
127	fluorescent tubes (OSRAM) with a 16:8 h light:dark cycle. Cells were harvested during the
128	exponential phase of growth, centrifuged at 3000 g for 10 min and resuspended in their
129	culture medium to a final chlorophyll (Chl) <i>a</i> concentration of 10 μ g Chl <i>a</i> mL ⁻¹ . The algae
130	were continuously stirred at 21°C under low continuous light. For oxygen (O ₂), Chl
131	fluorescence and thermoluminescence measurements, cells were dark-adapted 20 min prior
132	to measurement.
133	
134	Protein extraction and western-blot analysis
135	Cells were harvested during the exponential phase of growth as described above
136	and subsequently grinded in liquid nitrogen. The homogenized cells were resuspended in
137	preheated (60°C) extraction buffer (125 mM Tris/HCl (pH 6.8), 4% (w/v) SDS, 200 µM
138	PMSF and 100 mM DTT) and after heat treatment extracted with acetone. After wash
139	steps, the dried protein pellet was finally resuspended in extraction buffer. Total protein
140	was separated by SDS-PAGE using 12%. Proteins were transferred electrophoretically

141	onto a PVDF membrane (Hybond [™] -P, Amersham Biosciences UK Limited,
142	Buckinghamshire, England) and incubated with an antiserum against D1 (Anti-PsbA
143	global antibody, AS05 084, Agrisera, Sweden). Detection was performed using the
144	chemoluminescence detection system from Roche Diagnostics (BM Chemiluminescence
145	Blotting Substrate POD).
146	
147	Pigment extraction and analysis
148	Chl a amount was determined by spectrophotometry using the 90% acetone
149	extraction method. For pigment extraction cells were deposited on a filter and frozen in
150	liquid nitrogen. Pigments were extracted with a methanol:acetone (70:30, v/v) solution.
151	Pigment analysis was performed via HPLC as previously described (Lavaud et al. 2003).
152	Cell counts were performed using a Thoma hematocytometer.
153	
154	Spectroscopy and PSI reaction center (P700) concentration.
155	The absorption spectra were obtained at room temperature with a DW-2 Aminco
156	(American Instrument Co., USA) spectrophotometer, half-bandwith 3 nm, speed 2 nm s ⁻¹ ,
157	OD = 0 at 750 nm, 50% F/2 medium as a reference. P_{700} quantity relative to Chl <i>a</i> was
158	determined as described earlier (Lavaud et al. 2002a) with the DW-2 Aminco
159	spectrophotometer in dual beam mode (reference at 730 nm).
160	

161 *Thermoluminescence*

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162	Thermoluminescence patterns were measured with a self-made thermoluminometer
163	following the procedure previously described (Gilbert et al. 2004). Flashes were single turn-
164	over with duration of 25 μ s. Samples were adjusted to 20 μ g Chl <i>a</i> mL ⁻¹ for measurement.
165	
166	Oxygen (O_2) concentration and photosynthetic light-response (P/E) curves.
167	O ₂ concentration was measured with a DW1-Clark electrode (Hansatech Ltd.,
168	England) at 21°C. White light of adjustable intensity (measured with a PAR-sensor, Licor
169	inc., USA) was provided by a KL-1500 quartz iodine lamp (Schott, Mainz, Germany).
170	Cell culture samples were dark-adapted for 20 min before measurement. P/E curves were
171	obtained by illuminating a 2 mL sample during 5 min at various light intensities. A new
172	sample was used for each measurement. E_{K} , the irradiance for saturation of photosynthetic
173	O ₂ emission was estimated from P/E curves.
174	
175	Chlorophyll fluorescence induction kinetics and DCMU resistance
176	Chl a fluorescence induction kinetics were performed with two instruments: a
177	PEA-fluorometer (Walz, Effeltrich, Germany) for short time kinetics (up to 200 ms), which
178	allowed a classic OIJP analysis (see Fig. S4 for details), and a self-made 'continuous
179	light' fluorometer (Parésys et al. 2005) for long time kinetics (up to 100 s). Cells were
180	adjusted to a concentration of 5 μ g Chl <i>a</i> mL ⁻¹ and 20 μ g Chl <i>a</i> L ⁻¹ for the PEA- and the
181	self-made fluorometers, respectively.
182	DCMU resistance was evaluated measuring the inhibition of the PSII activity vs
183	increasing DCMU concentrations (see Fig. S4 for details). The kill curves with DCMU

184	and atrazine were performed by growing the cells on solid medium with increasing
185	concentrations of herbicides; growth conditions were the same as described before.
186	
187	Chlorophyll fluorescence yield
188	Chl fluorescence yield was monitored with a modified PAM-101 fluorometer (Walz,
189	Effeltrich, Germany) as described previously (Lavaud et al. 2002a). For each experiment, 2
190	mL were used. Sodium bicarbonate was added at a concentration of 4 mM to prevent any
191	limitation of the photosynthetic rate by carbon supply. When used, DCMU was incubated
192	with the cell suspension at the beginning of the dark-adaptation period. Fluorescence
193	parameters were defined as described in Fig. S4. 1-qP was used as a parameter to estimate
194	the fraction of reduced Q_A (Büchel and Wilhelm 1993) where qP is the photochemical
195	quenching of Chl fluorescence. The rate of linear electron transport was calculated as
196	ETR = Φ PSII x PDF x α x 0.5, where Φ PSII is the PSII quantum yield for
197	photochemistry, PDF is the irradiance, α is the PSII antenna size (equivalent to $1/I_{1/2}$ of
198	Y _{SS} , see below).
199	
200	$Oxygen (O_2)$ yield per flash
201	The relative O ₂ yield produced per flash during a sequence of single-turnover
202	saturating flashes (O_2 sequence) was measured polarographically at 21°C with a flash
203	electrode as described by Lavaud et al. 2002b. The flashes were separated by 500 ms
204	allowing the reopening of PSII RCs by reoxidation of Q_A , between each flash. The
205	procedures used to record and calculate the steady-state O_2 yiel per flash (Y_{SS} , an

206 evaluation of the number of O₂ producing PSII RCs relative to Chl a), the reciprocal of

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207	the half-saturating flash intensity of flash O_2 evolution saturation curves ($1/I_{1/2}$ of Y_{SS} , and
208	evaluation of the PSII antenna size), and the miss probability per PSII were the ones
209	described in Fig. S4.
210	
211	Results
212	Generating the Phaeodactylum tricornutum psbA mutants
213	In an attempt to establish stable plastid transformation in <i>P. tricornutum</i> we aimed
214	for allele replacement via homologous recombination. To minimize impact on the diatom
215	we decided to substitute the wild-type (WT) psbA gene with slightly modified versions
216	carrying alternative point mutations in codon 264 (Fig. S2B, green boxes). These
217	mutations lead to single amino acid substitutions that were previously reported to induce
218	herbicide resistance and a reduction of the electron transport within the photosystem II
219	reaction center (PSII RC) (Ohad and Hirschberg 1992, Oettmeier 1999). Sequencing of
220	the target region in several putative transformants revealed a variety of non-synonymous
221	(and in some cases additional synonymous) point mutations. In all experiments the
222	observed point mutations occurred apparently random and independently of the
223	respective vector sequence. None of the obtained resistant strains carried the same pair of
224	point mutations that was supposed to be introduced into psbA by the utilized
225	transformation vector (data not shown). Negative control experiments involving
226	exclusive selection without preceding transformation, and biolistic transformation
227	without vector DNA failed to generate resistant colonies. We sequenced 1000 bp regions
228	surrounding the $Q_{\rm B}$ pocket as well as coding and non-coding areas more distant to the
229	psbA locus without finding other mutations than the ones described here. Yet, we cannot

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230	exclude the possibility that additional mutations occurred at unknown loci. However, due
231	to the selection on DCMU, which specifically interacts with the Q_B pocket of the D1
232	protein, additional mutations at other loci are likely to be detrimental and therefore
233	selected against.
234	Although intriguing, this study is not focusing on the underlying molecular
235	mechanism leading to the elevated mutation rates in the <i>psbA</i> gene; it will be the focus of
236	a subsequent work. Instead, we characterized and compared for four selected mutants the
237	physiological effects of different amino acid substitutions in the D1 protein of the PSII
238	<u>RC</u> on the regulation of photosynthesis.
239	
240	Localization of the mutations in the D1 protein and herbicide resistance of the
241	Phaeodactylum tricornutum psbA mutants
242	The highly conserved D1 protein is part of the PSII RC in cyanobacteria and all
243	phyla of plastid containing photosynthetic eukaryotes (Fig. S2A). The quinone B (Q_B)
244	binding pocket is located between the DE helix and the transmembrane E helix of the D1
245	protein (Kern and Renger 2007). The functional relevance of the Q_B binding pocket (Fig.
246	<u>S2B</u>) is highlighted by an amino acid sequence similarity of 97-98% between pennate and
247	centric diatoms, and a similarity of ~ 90-93% between diatoms and members of the red
248	lineage, the green lineage, and even cyanobacteria (Fig. $\underline{S3}$). Sequencing the <i>psbA</i> genes
249	of the four mutant strains revealed point mutations within or near the Q_B binding pocket
250	(Fig. S2B, red squares). The mutant V219I featured an amino acid exchange (Val to Ile at
251	position 219) in transmembrane helix D. In mutant F255I a Phe was changed to Ile in

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252	helix DE close to the Q_B pocket. S264A carries a Ser to Ala substitution within the Q_B
253	pocket, and in L275W, Leu was changed to Trp in the helix E.
254	In comparison to the wild-type (WT) the competitive binding of the herbicide
255	DCMU to the Q _B pocket was altered to a different degree in all mutants (Fig. S4), among
256	which S264A featured the highest resistance (3000 fold). The level of resistance was
257	confirmed by growth curves in the presence of increasing concentrations of DCMU (not
258	shown). S264A was also highly resistant against the herbicide atrazin (500 fold).
259	
260	Photosystem and light-harvesting properties, and growth of the Phaeodactylum
261	tricornutum psbA mutants
262	At low light intensity (50 μ mol photons m ⁻² s ⁻¹), the pigment contents of all the
263	mutants and the WT cells were very similar although the mutants tended to accumulate
264	slightly more Chl <i>a</i> per cell (see Fig. S4). The concentrations of active PSII RCs per Chl
265	a, Y_{SS} and RC/CS ₀ , were higher in all the mutants but L275W (<u>Fig. S4</u>). The low
266	concentration for L275W was confirmed via western-blot analysis (Fig. $\underline{1}$). The molar
267	PSI:PSII ratio was similar in WT and mutants with the exception of L275W, for which
268	the ratio was higher (x 1.3) ratio. The PSII LHC (light-harvesting complex) antenna size
269	$(1/I_{1/2} \text{ of } Y_{SS})$, as well as E_K , the light intensity for saturation of photosynthesis were
270	lower in all the mutants, with the exception of V219I (Fig. S4).
271	We compared the physiological effects of the four mutations by measuring
272	thermoluminescence, flash oxygen (O ₂) yield emission (O ₂ sequence) and Chl a
273	fluorescence induction kinetics. WT cells showed the expected thermoluminescence
274	pattern with a strong B band (Fig. 2A) (Eisenstadt et al. 2008). While V219I showed the

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275	same pattern, in F255I and S264A the temperature of the maximal signal was shifted
276	from 22°C to about 7°C and had significantly lowered amplitude (Fig. 2A). The O ₂
277	sequences were highly damped in dark-adapted cells of F255I, S264A and L275W (Fig.
278	2B) due to an increase in the miss probability (Fig. S4). In addition, in S264A and to a
279	lesser extent in F255I (not shown), the O_2 production was increased at flash n°2 (due to
280	an increase of 10-20% of the S_1 dark state in S264A compared to the WT) while in
281	L275W, the maximum was at the flash n°4 instead of n°3. Chl a fluorescence induction
282	kinetics are shown in Fig. 2C and Fig. 2D. All the mutants showed higher J ($Q_A Q_B / Q_A$
283	Q_B^- state) and lower I ($Q_A^-Q_B^{-2-}$ state) phases (Fig. 2C and <u>Fig. S4</u>) reflecting an
284	impairment of the Q_A - Q_B electron transfer. The phenotype of V219I was the closest to
285	WT phenotype while F255I and L275W showed a significantly higher J phase (+ 23 and
286	57%, respectively). S264A showed a drastically increased (by 71%) and delayed J phase
287	and, in contrast to the other mutants, an increased I phase (see inset Fig. 2C and Fig. S4).
288	When recorded over a longer time scale (100 s) and at continuous illumination, the
289	pattern of the fluorescence induction kinetics of S264A and L275W was different (only
290	L275W is shown, Fig. 2D). In S264A and L275W, the amplitude of the I-45 ms peak
291	increased and the whole pattern of the kinetics was disturbed.
292	The F_0 Chl <i>a</i> fluorescence level was increased in all mutants (Fig. S4). Adding
293	DCMU (resulting in inhibition of electron transport between Q_A and Q_B) to WT cells
294	resulted in an increased F_0 (195 ± 6.5) comparable to S264A and L275W. When grown at
295	low light intensity (50 μ mol photons m ⁻² s ⁻¹) all mutants showed a maximum
296	photosynthetic efficiency of PSII (F_v/F_m , Fig. S4) which was similar to the WT cells, except
297	for L275W (- 19%). When measured at an equivalent irradiance, the effective PSII quantum

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298	yield (Φ PSII, <u>Fig. S4</u>) was the same for WT cells and V219I, but lower in the other
299	mutants. These values were in accordance with the steady-state electron transport rate per
300	PSII (ET ₀ /CS ₀ , <u>Fig. S4</u>). Addition of DCMU to the WT resulted in a decreased Φ PSII
301	(0.38), similar to that of S264A and L275W.
302	Only L275W showed a reduced (- 26%) growth rate (μ , Fig. S4) and final maximal
303	biomass (Fig. <u>\$5</u>). Although F255I and S264A reached the same final biomass with the
304	same growth rate as the WT, they showed a 24 h delay (see days 3 and 2, respectively, Fig.
305	<u>85</u>).
306	
307	Photosynthetic capacity of the Phaeodactylum tricornutum psbA mutants as a function of
308	the light intensity
309	The light intensity dependent impairment of the Q_A - Q_B electron transfer was
310	evaluated by measuring 1-qP, a fluorescence parameter which estimates the fraction of
311	reduced Q _A (Büchel and Wilhelm 1993). While 1-qP was similar in WT and in V219I, it
312	was highest in S264A and L275W (Fig. 3A). The difference in the extent of Q_A reduction
313	was also found at rather low light intensities (inset Fig. 3A) as indicated by the ratios of
314	the extent of the I-45 ms peak from the long-time fluorescence induction kinetics of
315	mutant versus WT (see Fig. 2D). In S264A and L275W, 1-qP reached saturation earlier
316	(between 250 and 400 μmol photons $m^{-2}~s^{-1}$) than in WT cells. In F255I, the extent of Q_A
317	reduction was higher than in WT up to a light intensity of 400 μ mol photons m ⁻² s ⁻¹ . The
318	direct consequences of the impaired Q_A - Q_B electron transfer were changed amplitudes of
319	the electron transport rate per PSII (ETR) as well as altered patterns of ETR as a function
320	of light intensity (Fig. 3B). The maximum ETR was decreased in all the mutants but to a

321 different extent, thus confirming the values for ET_0/CS_0 (Fig. S4). In contrast to WT and 322 the other mutants, ETR was already maximal in S264A and L275W at a light intensity of 323 250 µmol photons m⁻² s⁻¹; at this light intensity the extent of Q_A reduction was close to its 324 maximum (Fig. 3A).

- 325
- 326 **Discussion**

Three out of the four *psbA* mutants showed a phenotype clearly distinct from the WT (see Fig. 4). Obviously, the observed amino acid substitutions hold implications for the phenotype of the mutants. The phenotypic effects described in this study allow various insights into the functionality of mutated residues or domains within the D1 protein.

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333 A mutation which slightly affects the photosynthetic efficiency: V219I

In response to the slightly increased reduction state of Q_A (+ 10%) and the

decreased (about 5%) ETR per PSII, in V219I the number of PSII RCs was increased (14

to 22%, depending on the method) to maintain a photosynthetic activity similar to the WT

as reflected also by its growth pattern. Hence, the exchange of Val to Ile at the position

338 219 in the helix D appears to be too distant from the Q_B binding pocket to significantly

disturb the electron transport within the PSII RC in *P. tricornutum*.

340

341 Effects of mutations within the Q_B binding pocket: F255I and S264A

342 The residues Phe_{255} and Ser_{264} bind the head group of Q_B (Kern and Renger 2007). 343 The electron transport between Q_A and Q_B in F255I was significantly impaired (Fig. 4)

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344	slowing down the reoxidation of Q_A^- as illustrated by the increased $Q_A Q_B^-/Q_A^-Q_B^-$ state. It
345	was especially visible with the pattern of thermoluminescence which resembles the one
346	reported in <i>P. tricornutum</i> for WT cells treated with DCMU (abolishment of the B band
347	and increase of the Q band) (Eisenstadt et al. 2008). Backward electron transfer from Q_B^-
348	to Q_A , as illustrated by an enhanced F_0 in photochemically inactive PSII RCs (Xiong et
349	al. 1997), might partially explain the increased concentration of Q_A^- . As a consequence,
350	the miss probability of the S state-cycle was increased, the S ₁ state was stabilized
351	(Perewoska et al. 1994) and the lifetimes of the redox states S_2 and S_3 increased (Gleiter
352	et al. 1992), indicating a disturbed OEC operation. In order to compensate the decreased
353	photochemistry of PSII, in F255I the number of PSII RCs increased (Fig. 4), reflected by
354	a slight increase of Chl <i>a</i> per cell as also reported for higher plants (Srivasatava et al.
355	1994). Nevertheless, the overall amount of pigments per Chl a did not change, thus the
356	antenna size per PSII decreased leading to a similar increase in E_K . Decreasing the PSII
357	antenna size is known to be a straightforward way to relief from high excitation pressure
358	on PSII due to a slowed down electron flow within the PSII RC because of mutations,
359	herbicides, environmental stress, or other factors. Similarly, Wagner et al. (2006)
360	suggested that in <i>P. tricornutum</i> an increased number of photosynthetic units together
361	with decreased size of these units might allow maximization of photochemistry at
362	different light regimes which might be the case in mutants F255I and S264A. In spite of
363	all these changes, the potential for photochemistry, qP, was decreased at intermediary
364	irradiances (up to 400 μ mol photons m ⁻² s ⁻¹). At high light intensities, the ETR was
365	reduced (Fig. 4) reflecting the decrease of the PSII antenna size and of Φ PSII.

366	S264A showed a more drastic reaction compared to F255I regarding the Q_A^{-1}
367	reoxidation, the electron back transfer Q_B^- to Q_A , but also the Q_B^-/Q_B^{-2-} reoxidation
368	(increased $Q_A^- Q_B^{2-}$ state) (Fig. 4). Consequently, the operation of the OEC S-state cycle
369	was strongly disturbed similar to the pattern of the fluorescence induction kinetics,
370	illustrating the consequence of the modified Q_A - Q_B redox state on the whole electron
371	transport chain and especially on the redox state of the plastoquinone (PQ) pool (Lazar
372	2006, Papageorgiou et al. 2007). As F255I, S264A reacted by increasing the PSII
373	number. qP was largely diminished which usually reflects accumulation of dysfunctional,
374	highly reduced PSII RCs. It led to a decrease in ETR at all light intensities. Both qP and
375	ETR were saturated at a much lower irradiance than the WT. The exchange of Ser to Ala
376	probably modified the spatial arrangement of the Q_B pocket (Gleiter et al. 1992,
377	Perewoska et al. 1994), as illustrated by the high DCMU resistance, and greatly impaired
378	not only the redox state of Q_B but the binding of Q_B itself (Della Chiesa et al. 1997).
379	
380	Effects of a mutation close to the non-heme iron binding site: L275W
381	Leu_{275} is close to one of the Histidines binding the non-heme iron atom (His ₂₇₂ in
382	helix E, grey bar in Fig. S2), as well as at nearly equal distance between Q_A and Q_B (Kern
383	and Renger 2007). The effect of the L275W mutation on the photosynthetic ability per
384	PSII was similar to the point mutation S264A (Fig. 4) but showed a much disturbed OEC
385	operation and a reduction of Q_A which was already high (1.2-1.3 times the WT values) at
386	lower light intensities than used for growing cells. Ultimately, L275W showed a
387	decreased growth rate under low light as well as the inability to reach the same final
388	maximal biomass. The main difference compared to the other mutants was the reduced

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389	amount of active PSII (Fig. 4), demonstrated by a disturbed D1 repair cycle. Mutations
390	close to or within the Q_B pocket have been reported to modify the D1 turnover either by
391	accelerating its damage and/or by inhibiting its proteolysis and/or synthesis (Della Chiesa
392	et al. 1997, Nishiyama et al. 2006). Thus, it is very likely that in L275W there is a mixed
393	population of active and inactive PSII, even at low light intensities (Mohanty et al. 2007),
394	which is supported by the high F_0 level and the lowest F_v/F_m . In contrast to the other
395	mutants, L275W responded to the point mutation by modifying the architecture of the
396	photosynthetic apparatus as illustrated by the increase of the PSI:PSII stochiometry (Fig.
397	4). This attempt to maintain a reasonable photosynthetic activity might lead to an
398	increased capacity for PSI cyclic electron flow as in higher plants that show a deficient
399	linear electron transport (Kotakis et al. 2006).
400	In a series of papers (reviewed in van Rensen et al. 1999), Govindjee and co-
401	workers showed that the exchange of the residue Leu_{275} significantly perturbs the Q_A -Fe-
402	Q_B structure, the protonation of Q_B^{2-} (Xiong et al. 1997) and subsequently the PQ redox
403	state. It is thus likely that the phenotype of L275W is due to the close vicinity of the point
404	mutation to both the Q_B pocket and the non-heme iron atom binding sites which
405	functionally affects the properties of both the Q_A and Q_B pockets (Vermaas et al. 1994).
406	
407	Effects of similar mutations in cyanobacteria and green algae
408	The effects of the mutations described here for the diatom <i>P. tricornutum</i> are
409	similar to effects of the same mutations reported in other photosynthetic organisms. For
410	example, it has also been concluded that in Chlamydomonas reinhardtii (Erickson et al.
411	1989) the V219I amino acid substitution does not significantly disturb the electron

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412	transport within the PSII RC. Also, the effects of the F255I, S264A and L275W
413	mutations have been described in cyanobacteria (Synechocystis and Synechococcus) and
414	C. reinhardtii (Erickson et al. 1989, Etienne 1990, Gleiter et al. 1992, Kless et al. 1994,
415	Perewoska et al. 1994). Remarkably, the S264A induced DCMU resistance was much
416	higher in <i>P. tricornutum</i> than in all previously studied organisms (Gleiter et al. 1992).
417	
418	Conclusion
419	Our results illustrate that not only the substitution loci but also the nature of the
420	exchanged amino acids are essential in modifying the spatial arrangement and properties
421	of the D1 protein (Kless and Vermaas 1994). Ultimately, such structural changes,
422	especially in the Q_B binding pocket, are defining the electron transport rate within the
423	PSII RC (Lardans et al. 1998, Oettmeier 1999). The fact that photosynthesis is impaired
424	at different levels in the <i>P. tricornutum psbA</i> mutants described here (see Fig. 4) provides
425	a unique opportunity to further study the regulation of photosynthesis in diatoms.
426	
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437	
438	References
439	Allen, A. E., Vardi, A. & Bowler, C. 2006. An ecological and evolutionary context for
440	integrated nitrogen metabolism and related signaling pathways in marine diatoms. Curr.
441	Opin. Plant Biol. 9:264-73
442	Allen, A. E., LaRoche, J., Maheswari, U., Lommer, M., Schauer, N., Lopez, P. J.,
443	Finazzi, G., Fernie, A. R. & Bowler, C. 2008. Whole-cell response of the pennate diatom
444	Phaeodactylum tricornutum to iron starvation. Proc. Natl. Acad. Sci. USA 105:10438-
445	10443
446	Armbrust, E. V., Berges, J. A., Bowler, C., Green, B. R., Martinez, D., Putnam, N. H.,
447	Zhou S., Allen, A. E., Apt, K. E., Bechner, M., Brzezinski, M. A., Chaal, B. K., Chiovitti,
448	A., Davis, A. K., Demarest, M. S., Detter, J. C., Glavina, T., Goodstein, D., Hadi, M. Z.,
449	Hellsten, U., Hildebrand, M., Jenkins, B. D., Jurka, J., Kapitonov, V. V., Kröger, N., Lau,
450	W. W. Y., Lane, T. W., Larimer, F. W., Lippmeier, J. C., Lucas, S., Medina, M.,
451	Montsant, A., Obornik, M., Parker, M. S., Palenik, B., Pazour, G. J., Richardson, P. M.,
452	Rynearson, T. A., Saito, M. A., Schwartz, D. C., Thamatrakoln, K., Valentin, K., Vardi,
453	A., Wilkerson, F. P. & Rokhsar, D. S. 2004. The genome of the diatom Thalassiosira
454	pseudonana: Ecology, evolution and metabolism. Science 306:79-86
455	Bowler, C., Allen, A. E., Badger, J. H., Grimwood, J., Jabbari, K., Kuo, A., Maheswari,
456	U., Martens, C., Maumus, F., Otillar, R. P., Rayko, E., Salamov, A., Vandepoele, K.,
457	Beszteri, B., Gruber, A., Heijde, M., Katinka, M., Mock T., Valentin, K., Vérret, F.,

458	Berges, J.A,. Brownlee, C., Cadoret, J.P., Chiovitti, A., Choi, C.J., Coesel, S., De
459	Martino, A., Detter, J. D., Durkin, C., Falciatore, A., Fournet, J., Haruta, M., Huysman,
460	M., Jenkins, B. D., Jiroutova, K., Jorgensen, R. E., Joubert, Y., Kaplan, A., Kroeger, N.,
461	Kroth, P. G., La Roche, J., Lindquist, E., Lommer, M., Martin-Jézéquel, V., Lopez, P. J.,
462	Lucas, S., Mangogna, M., McGinnis, K., Medlin, L. K., Montsant, A., Oudot-Le Secq, M.
463	P., Napoli, N., Obornik, M., Petit, J. L., Porcel, B. L., Poulsen, N., Robison, M.,
464	Rychlewski, L., Rynearson, T. A., Schmutz, J., Schnitzler Parker, M., Shapiro, H., Siaut,
465	M., Stanley, M., Sussman, M. R., Taylor, A., Vardi, A., von Dassow, P., Vyverman, W.,
466	Willis, A., Wyrwicz, L. S., Rokhsar, D.S., Weissenbach, J., Armbrust, E. V., Green, B.
467	R., Van de Peer, Y., Grigoriev, I. V. (2008) The Phaeodactylum genome reveals the
468	evolutionary history of diatom genomes. Nature 455: online, doi:10.1038/nature07410.
469	Büchel, C. & Wilhelm, C. 1993. In vivo analysis of slow chlorophyll fluorescence
470	induction kinetics in algae: progress, problems and perspectives. Photochem. Photobiol.
471	58:137-48
472	Della Chiesa, M., Friso, G., Deak, Z., Vass, I., Barber, J. & Nixon, P. 1997. Reduced
473	turnover of the D1 polypeptide and photoactivation of electron transfer in novel herbicide
474	resistant mutants of Synechocystis sp. PCC 6803. Eur. J. Biochem. 248:731-40
475	Doyle, J. J. & Doyle, J. L. 1990. A rapid total DNA preparation procedure for fresh plant
476	tissue. Focus 12:13-5
477	Eisenstadt, D., Ohad, I., Keren, I. & Kaplan, A. 2008. Changes in the photosynthetic
478	reaction center II in the diatom Phaeodactylum tricornutum result in non-photochemical
479	fluorescence quenching. Environ. Microbiol. 10:1997-2007

|

Journal of Phycology

- 480 Erickson, J. M., Pfister, K., Rahire, M., Togasaki, R. K., Mets, L. & Rochaix, J.-D. 1989.
- 481 Molecular and biophysical analysis of herbicide-resistant mutants of *Chlamydomonas*
- 482 reinhardtii: Structure-function relationship of the photosystem II D1 polypeptide. Plant
- 483 *Cell* 1:361-71
- 484 Etienne, A.-L., Ducruet, J.-M., Ajlani, G. & Vernotte, C. 1990. Comparative studies on
- 485 electron transfer in photosystem II of herbicide-resistant mutants from different
- 486 organisms. *Biochim. Biophys. Acta* 1015:435-40
- 487 Falkowski, P. G., Katz, M. E., Knoll, A. H., Quigg, A., Raven, J. A., Schofield, O. &
- 488 Taylor, F. R. J. 2004. The evolution of modern phytoplankton. *Science* 305:354-60
- 489 Fufezan, C., Gross, C. M., Sjödin, M., Rutherford, A. W., Krieger-Liszkay, A. &
- 490 Kirilovsky, D. 2007. Influence of the redox potential of the primary quinone electron
- 491 acceptor on photoinhibition in photosystem II. J. Biol. Chem. 282:12492-502
- 492 Gilbert, M., Wagner, H., Weingart, I., Nieber, K., Tauer, G., Bergmann, F., Fischer, H. &
- 493 Wilhelm, C. 2004. A new type of thermoluminometer: A highly sensitive tool in applied
- 494 photosynthesis research and plant stress physiology. J. Plant Physiol. 161:641-51
- 495 Gleiter, H. M., Ohad, N., Kolke, H., Hirschberg, J., Renger, G. & Inoue, Y. 1992.
- 496 Thermoluminescence and flash-induced oxygen yield in herbicide resistant mutants of the
- 497 D1 protein in Synechococcus PCC7942. Biochim. Biophys. Acta 1140:135-43
- 498 Guillard, R. R. R. & Ryther, J. H. 1962. Studies of marin planktonic diatoms. 1. C. nana
- 499 (Hustedt) and D. confervacea (Cleve). Gran. Can. J. Microbiol. 8:229-38
- 500 Ishikawa, Y., Nakatani, E., Henmi, T., Ferjani, A., Harada, Y., Tamura, N. & Yamamoto,
- 501 Y. 1999. Turnover of the aggregates and cross-linked products of the D1 protein

- 502 generated by acceptor-side photoinhibition of photosystem II. Biochim. Biophys. Acta
- 503 1413:147-58
- 504 Keeling, P. 2004. A brief history of plastids and their hosts. *Protist* 155:3-7
- 505 Kern, J. & Renger, G. 2007. Photosystem II: Structure and mechanism of the
- 506 water:plastoquinone oxidoreductase. *Photosynth. Res.* 94:183-202
- 507 Kless, H., Oren-Shamir, M., Malkin, S., McIntosh, L. & Edelman, M. 1994. The *D-E*
- 508 region of the D1 protein is involved in multiple quinone and herbicide interactions in
- 509 photosystem II. *Biochemistry* 33:10501-07
- 510 Kless, H. & Vermaas, W. 1994. Many combinations of amino acid sequences in a
- 511 conserved region of the D1 protein satisfy photosystem II function. J. Mol. Biol. 246:120-

- 513 Kotakis, C., Petropoulou, Y., Stamatakis, K., Yiotis, C. & Manetas, Y. 2006. Evidence
- 514 for active cyclic electron flow in twig chlorenchyma in the presence of an extremely
- 515 deficient linear electron transport activity. *Planta* 225:245-53
- 516 Kroth, P. G. 2007. Genetic transformation a tool to study protein targeting in diatoms.
- 517 In Van der Giezen, M. ed Methods in Molecular Biology Protein Targeting Protocols.
- 518 Humana Press, Totowa, USA, pp. 257-68
- 519 Kroth, P. G., Chiovitti, A., Gruber, A., Martin-Jézéquel, V., Mock, T., Schnitzler Parker,
- 520 M., Stanley, M. S., Kaplan, A., Caron, L., Weber, T., Maheswari, U., Armbrust, V. E. &
- 521 Bowler, C. 2008. A model for carbohydrate metabolism in the diatom *Phaeodactylum*
- 522 *tricornutum* deduced from comparative whole genome analysis. *PLoS One* 3:e1426.
- 523 Lardans, A., Förster, B., Prasil, O., Falkowski, P. G., Sobolev, V., Edelman, M., Osmond,
- 524 C. B., Gillham, N. W. & Boynton, J. E. 1998. Biophysical, biochemical, and

Journal of Phycology

- 525 physiological characterization of Chlamydomonas reinhardtii mutants with amino acid
- 526 substitutions at the Ala²⁵¹ residue in the D1 protein that result in varying levels of
- 527 photosynthetic competence. J. Biol. Chem. 273:11062-91
- 528 Lavaud, J. 2007. Fast regulation of photosynthesis in diatoms: mechanisms, evolution and
- 529 ecophysiology (a review). Funct. Plant Sci. Biotech. 1:267-87
- 530 Lavaud, J. & Kroth, P. 2006. In diatoms, the transthylakoid proton gradient regulates the
- 531 photoprotective non-photochemical fluorescence quenching beyond its control on the
- 532 xanthophyll cycle. *Plant Cell Physiol.* 47:1010-6
- 533 Lavaud, J., Rousseau, B. & Etienne, A.-L. 2003. Enrichment of the light-harvesting
- 534 complex in diadinoxanthin and implications for the nonphotochemical quenching
- fluorescence quenching in diatoms. *Biochemistry USA* 42:5802-8
- 536 Lavaud, J, van Gorkom, H. & Etienne, A.-L. 2002b. Photosystem II electron transfer
- 537 cycle and chlororespiration in planktonic diatoms. *Photosynth. Res.* 74:51-9
- 538 Lavaud, J., Rousseau, B., van Gorkom, H. & Etienne, A.-L. 2002a. Influence in the
- 539 diadinoxanthin pool size on photoprotection in the marine planktonic diatom
- 540 Phaeodactylum tricornutum. Plant Physiol. 129:1398-406
- 541 Lazar, D. 2006. The polyphasic chlorophyll *a* fluorescence rise measured under high
- 542 intensity of exciting light. *Funct. Plant Biol.* 33:9-30
- 543 Long, S., Humphries, S. & Falkowski, P. 1994. Photoinhibition of photosynthesis in
- 544 nature. Ann. Rev. Plant Physiol. Plant Mol. Biol. 45:633-62
- 545 MacIntyre, H. L., Kana, T. M. & Geider, R. J. 2000. The effect of water motion on short-
- 546 term rates of photosynthesis by marine phytoplankton. *TRENDS Plant Sci.* 5:12-7
- 547 Mann, D. G. 1999. The species concept in diatoms. *Phycologia* 38:437-495

- 548 Mohanty, P., Allakhverdiev, S. I. & Murata, N. 2007. Application of low temperatures
- 549 during photoinhibition allows characterization of individual steps in photodamage and the
- repair of photosystem II. *Photosynth. Res.* 94:217-24
- 551 Nishiyama, Y., Allakhverdiev, S. I. & Murata, N. 2006. A new paradigm for the action of
- 552 reactive oxygen species in the photoinhibition of photosystem II. Biochim. Biophys. Acta
- 553 1757:742-9
- 554 Oettmeier, W. 1999. Herbicide resistance and supersensitivity in photosystem II. Cell.
- 555 Mol. Life Sci. 55:1255-77
- 556 Ohad, N. & Hirshberg, J. 1992. Mutations in the D1 subunit of photosystem II distinguish
- 557 between quinone and herbicide binding sites. *Plant Cell* 4:273-82
- 558 Papageorgiou, G. C., Tsimilli-Michael, M. & Stamatakis, K. 2007. The fast and slow
- 559 kinetics of chlorophyll *a* fluorescence induction in plants, algae and cyanobacteria: a
- 560 viewpoint. Photosynth. Res. 94:275-90
- 561 Parésys, G., Rigart, C., Rousseau, B., Wong, A. W. M., Fan, F., Barbier, J.-P. & Lavaud,
- 562 J. 2005. Quantitative and qualitative evaluation of phytoplankton populations by
- 563 trichromatic chlorophyll fluorescence excitation with special focus on cyanobacteria.
- 564 *Water Res.* 39:911-21
- 565 Perewoska, I., Etienne, A.-L., Miranda, T. & Kirilovsky, D. 1994. S₁ destabilazation and
- 566 higher sensitivity to light in metribuzin-resistant mutants. *Plant Physiol.* 104:235-45
- 567 Sarthou, G., Timmermans, K. R., Blain, S. & Tréguer, P. 2005. Growth physiology and
- fate of diatoms in the ocean: a review. J. Sea Res. 53:25-41
- 569 Srivasatava, A., Mohanty, P. & Bose, S. 1994. Alterations in the excitation energy
- 570 distribution in *Synechococcus* PCC 7942 due to prolonged partial inhibition of

|

Journal of Phycology

571	photosystem II. Comparison between inhibition caused by (a) presence of PSII inhibitors,
572	(b) mutation in the D1 polypeptide of PSII. Biochim. Biophys. Acta 1186:1-11
573	van Rensen, J. J. S., Xu, C. & Govindjee 1999. Role of bicarbonate in photosystem II, the
574	water-plastoquinone oxido-reductase of plant photosynthesis. Physiol. Plant. 105:585-92
575	Vass, I., Styring, S., Hundal, T., Koivuniemi, A., Aro, EM. & Andersson, B. 1992.
576	Reversible and irreversible intermediates during photohinibition of photosystem II: Stable
577	reduced Q _A species promote chlorophyll triplet formation. Proc. Natl. Acad. Sci. USA
578	89:1408-12
579	Vermaas, W., Vass, I., Eggers, B. & Styring, S. 1994. Mutation of a putative ligand to the
580	non-heme iron in Photosystem II: Implications for Q(A) reactivity, electron transfer, and
581	herbicide binding. Biochim. Biophys. Acta 1184:263-72
582	Wagner, H., Jakob, T. & Wilhelm, C. 2006. Balancing the energy flow from captured
583	light to biomass under fluctuating light conditions. New Phytol. 169:95-108
584	Wilhelm, C., Büchel, C., Fisahn, J., Goss, R., Jakob, T., LaRoche, J., Lavaud, J., Lohr,
585	M., Riebesell, U., Stehfest, K. & Kroth, P. 2006. The regulation of carbon and nutrient
586	assimilation in diatoms is significantly different from green algae. Protist 157:91-124
587	Xiong, J., Hutchison, R. S., Sayre, R. T. & Govindjee 1997. Modification of the
588	photosystem II acceptor side function in a D1 mutant (arginine-269-glycine) of
589	Chlamydomonas reinhardtii. Biochim. Biophys. Acta 1322:60-76

590	Legends

591

592 Fig. 1: Western-blot of the D1 protein of the PSII reaction center of P. tricornutum wildtype (WT) and the *psbA* mutant L275W cells. Cells were grown at 50 μ mol photons m⁻² s⁻ 593 594 ¹. Bands representing D1 degradation products of 23 kDa and the cross-link products of 595 around 83 kDa also resulting from D1 degradation (Ishikawa et al. 1999) were found in a 596 larger amount in L275W but not in the WT. 597 598 Fig. 2: A-Thermoluminescence emission of dark-adapted cells of *P. tricornutum* wild-599 type (WT) and the *psbA* mutants V219I / F255I / S264A. The characteristic emission 600 bands at 7°C (Q) and 22°C (B) are shown; they reflect the recombination states of the 601 PSII reaction center S_2Q_A and $S_{2/3}Q_B$ and the redox potential of Q_A and Q_B , respectively 602 (Gilbert et al. 2004; Eisenstadt et al. 2008). Curves are average of three measurements. B- O_2 production in a series of single-turnover flashes (O_2 sequences) by dark-adapted cells 603 604 of *P. tricornutum* wild-type (WT) and of the two *psbA* mutants S264A and L275W, as 605 measured via a flash electrode. The pattern of the O₂ sequence for V219I resembled the 606 one of the WT, and the pattern of F255I resembled the one of S264A with less 607 pronounced features. See Fig. S4 for a detailed description. C-/D-Chl a fluorescence 608 induction kinetics reflect quantum yield changes of Chl a fluorescence as a function of 609 the illumination duration, which relates to both excitation trapping in PSII and the 610 ensuing photosynthetic electron transport; C- Short-time kinetics recorded via PEA 611 fluorometer from dark-adapted cells of P. tricornutum wild-type (WT) and the four psbA 612 V219I / F255I / S264A / L275W mutants. The letters O, J, I P, H, G refer to the phases of

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613	the kinetics (Lazar 2006); D- Long-time kinetics from dark-adapted cells of WT and
614	L275W (same pattern for S264A) as recorded with a self-made 'continuous light'
615	fluorometer. The arrow indicates the first peak (I phase at 45 ms) which amplitude
616	reflects the redox state of Q_A (Lavaud and Kroth 2006). In diatoms, the classic P peak is
617	divided into two peaks H and G (Lazar 2006, Lavaud and Kroth, 2006).
618	
619	Fig. 3: Chl <i>a</i> fluorescence parameters as recorded with a PAM-fluorometer for the wild-
620	type (WT) and the four <i>psbA</i> V219I / F255I / S264A / L275W mutants of <i>P. tricornutum</i>
621	cells as a function of a light intensity gradient from darkness (0 μ mol photons m ⁻² s ⁻¹) to
622	the equivalent of full sunlight in nature (2000 μ mol photons m ⁻² s ⁻¹ , Long et al. 1994).
623	The illumination duration was 5 min; a new sample was used for each irradiance
624	treatment. A- 1-qP estimates the fraction of reduced Q_A (Büchel and Wilhelm 1993).
625	Inset: Ratio mutants vs WT of the amplitude of the I-45 ms peak (see Fig. 2D) up to 100
626	μ mol photons m ⁻² s ⁻¹ . B- ETR is the rate of linear electron transport. See the materials and
627	methods section and Fig. $\underline{S4}$ for details about the calculations of these parameters. Values
628	are average \pm SD of three to four measurements.
629	
630	Fig. 4: Diagram of the influence of mutations on the photosynthetic apparatus of the <i>psbA</i>
631	V219I / F255I / S264A / L275W mutants of P. tricornutum in comparison to the WT
632	situation. Left: the electron pathways within the PSII reaction center, right: the
633	architecture of the photosystems as a function of WT situation (PSI:PSII 1:2), arrow up,
634	increased value (the true value is given in-between brackets); arrow down, decrease; flat
635	arrow, no change. Symbols: red star, mutation; size of Q_A^{-}/Q_B^{-} , concentration of Q_A^{-}/Q_B^{-} ;

- thickness of the e⁻ arrows, value of the ETR_{max} and of the Q_B to Q_A back-transfer; dotted 636
- 637 feature of the OEC arrow, proportion of the disturbance of the OEC operation.
- 638 Abbreviations: E_K , light intensity for saturation of photosynthesis; e^{-} , electrons; LHC,
- 639 light-harvesting antenna complex; OEC, oxygen evolving complex; PSII/PS I RC,
- 640 photosystem I/photosystem II reaction center; PSI:PSII, molar photosystem
- 641 stoichiometry; Q_A and Q_B , quinones. See the text for a more detailed description.
- 642
- 643



Figure 1 117x117mm (600 x 600 DPI)





Figure 2





Figure 3 249x126mm (600 x 600 DPI)

υ x 60L



Figure 4

Supplementary File S1

Construction of the pGEM-T D1 transformation vectors



Using the primers P-psbA196-5' (5'-CTGTTGCAGGTTCTTTATTATATGG-3') and PpsbA990-3' (5'-TACTTCCATACCTAAATCAGCACGG-3') a 795 bp fragment of the plastid encoded *Phaeodactylum tricornutum* gene *psbA* was amplified following standard PCR procedures (Sambrook et al. 1995) and subsequently ligated into the insertion site of the commercially available TA cloning vector pGEM-T according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA).

The resulting vector pGEM-T D1 was subject to site directed mutagenesis substituting the codon 264 (Ser) with four alternative codons that encode the amino acids Ala

(pGEM-T D1-S264A), Gly (pGEM-T D1-S264G), Pro (pGEM-T D1-S264P), and Thr (pGEM-T D1-S264T). Moreover, a *BssSI* recognition site was altered by a silent T to A nucleotide substitution in codon 268 (not altering the encoded amino acid sequence). Site directed mutagenesis was performed via round circle PCR using he primers D1-792-fw (5'-CGTTTAATCTTCCAATACGCTXXXTTTAACAACTCaCGTGC-3') and D1-792rev (5'- GCACGTGAGTTGTTAAAXXXAGCGTATTGGAAGATTAAACG-3') and the pGEM-T D1 vectors as template. The XXX within the primer sequence refers to the altered codon (compared to the wild-type (WT) sequence) leading to the desired amino acid substitution. The small letter a in the D1-792-fw sequence indicates the second, conservative point mutation. Turbo-Pfu Polymerase (Stratagene, La Jolla, USA) was used for the round circle PCR reaction. The resulting products were treated with *Dpn*I for selective degradation of all non-modified (hence methylated) parent strands and subsequently transformed into chemically competent E. coli XL-1. The four resulting constructs served as transformation vectors in our attempts to transform the plastid genome of the diatom.

Supplementary File S2

Multiple sequence alignments and phylogenetic reconstruction

The strong selection pressure acting on *psbA* led to a high degree of sequence conservation that allows reconstructing a phylogeny of photosynthetic organisms based on the PsbA (D1) sequence that agrees well with more sophisticated phylogenetic reconstructions using more than one molecular marker (Delwiche 1999, Rodriguez-Ezpeleta et al. 2005).





A- Phylogenetic tree (neighbor joining, 100 fold bootstrapping) of D1 (PsbA) reconstructed from the amino acid sequence alignment (MUSCLE algorithm (Edgar 2004) of 35 individual protein sequences (see below for complete list). Although reflecting the current knowledge on plastid evolution in photosynthetic eukaryotes the purpose of this phylogenetic reconstruction is not to draw evolutionary conclusions (few branches have bootstrap support of less than 50), but rather intends to highlight the conservation level of D1 and to list the species included in our analysis of the sequence conservation of the Q_B binding pocket. Three groups consisting of green algae, red algae, and glaucophytes directly derive from the primary endocytobiosis between a cyanobacterium and a eukaryotic heterotrophic cell. Euglenozoans and chlorarachniophytes are derived from secondary endocytobiosis between a green alga and a eukaryotic host cell. Secondary endocytobiotic engulfment of a red alga by a eukaryotic host gave rise to the heterokonts (or stramenopiles), cryptophytes, haptophytes and dinoflagellates (Delwiche 1999, Cavalier-Smith 2000).

B- Sequence alignment of the Q_B pocket within the D1 protein. Light grey boxes, D, DE and E helices; dark grey bars, His₂₁₅ and His₂₇₂ which bind a non-heme iron atom; red squares, localization of the point mutations (from left to right) V219I / F255I / S264A / L275W in *Phaeodactylum tricornutum psbA* mutants; light green boxes, amino acid substitutions to be transferred by the respective transformation vector. The conservation plot below the alignment shows the degree of conservation (in %) for every individual site. The blot was inferred from an amino acid sequence alignment of D1 proteins from the 35 species listed in the phylogenetic tree above (Fig S2A, or see complete list below). Synechocystis, *Synechocystis* sp. PCC6803 (cyanobacteria, NC_000911);

Synechococcus WH7803 (cyanobacteria, CT971583); C. reinhardtii, Chlamydomonas
reinhardtii (Chlorophytes, AF396929); A. thaliana, Arabidospsis thaliana
(Embryophytes, CAB10554), C. merolae, Cyanidioschyzon merolae 10D (Rhodophytes, BAC76132); T. pseudonana, Thalassiosira pseudonana (Heterokontophytes, Bacillariophyceae, EF067921); O. sinensis, Odontella sinensis (Heterokontophytes, Bacillariophyceae, CAA91657); P. tricornutum, Phaeodactylum tricornutum
(Heterokontophytes, Bacillariophyceae, AY864816).

Methods:

All bioinformatic operations were performed using tools provided by the bioinformatic software package CLC Combined Workbench, Version 3.6.1 (CLC bio, Aarhus, Denmark). The phylogenetic tree for the D1 (PsbA) protein was reconstructed from an amino acid sequence alignment of entire D1 protein sequences from 35 different species (Fig. S3). For the alignment of protein sequences the MUSCLE algorithm was used (standard settings, maximal 16 iterations). The neighbor joining tree was reconstructed from the resulting alignment (data not shown), the value for the number of bootstrap replicates was set to 100. The tree was rooted above the common ancestor of the cyanobacteria. From the same alignment a conservation blot was inferred. Therefore the graphical alignment settings of the CLC Combined Workbench were set to 'Line plot'. For Fig S2B, amino acid sequences of the quinone B (Q_B) pocket (amino acid position 189 to 298) from eight species (Fig. S3) plus the translated sequencing results from the four mutant strains were aligned using the MUSCLE algorithm (standard settings, maximal 16 iterations).

References:

Cavalier-Smith, T. 2000. Membrane heredity and early chloroplast evolution. *Trends Plant Sci.* 5:174-82

Delwiche, C. F. 1999. Tracing the thread of plastid diversity through the tapestry of life. *Am. Nat.* 154:S164-77

Rodriguez-Ezpeleta, N., Brinkmann, H., Burey, S. C., Roure, B., Burger, G., Löffelhardt, W., Bohnert, H. J., Philippe, H., Lang, F. B. 2005. Monophyly of primary photosynthetic eukaryotes: Green plants, red algae and glaucophytes. Curr. Biol. 15:1325-1330

NCBI accession numbers of PsbA (D1) sequences (no particular order).

All listed *psbA* genes were used for the MUSCLE amino acid alignment of the entire protein sequence, from which the phylogenetic tree (Fig. S2A) was reconstructed. The conservation plot (Fig S4) has been derived from the same alignment. A subset (*) of the *psbA* genes listed was used for the MUSCLE amino acid alignment of the Q_B pocket (Fig S2B). From another subset ([†]) the pairwise comparison of amino acid similarities and distances was inferred (Fig. S4).

ACCESSION CP000576 ORGANISM **Prochlorococcus marinus str. MIT 9301** Bacteria; Cyanobacteria; Prochlorales; Prochlorococcaceae; Prochlorococcus.

ACCESSION NC_000911 ORGANISM **Synechocystis sp. PCC 6803***[†] Bacteria; Cyanobacteria; Chroococcales; Synechocystis.

ACCESSION CT971583 ORGANISM **Synechococcus sp. WH 7803***[†] Bacteria; Cyanobacteria; Chroococcales; Synechococcus.

ACCESSION BA000045 ORGANISM **Gloeobacter violaceus PCC 7421** Bacteria; Cyanobacteria; Gloeobacteriaes; Gloeobacter.

ACCESSION ORGANISM

Z00044 S54304 Nicotiana tabacum

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; asterids; lamiids; Solanales; Solanaceae; Nicotianoideae; Nicotianeae; Nicotiana.

ACCESSION

X86563 Zea mays

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;

ACCESSION ORGANISM

ORGANISM

EF044213 **Coffea arabica**

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;

ACCESSION

ORGANISM

AY228468 Dinus konsion

ORGANISM **Pinus koraiensis** Eukarvota: Viridiplantae: Streptophyta: Em

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Coniferopsida; Coniferales; Pinaceae; Pinus; Strobus.

ACCESSION AY522329

Oryza sativa (indica cultivar-group)

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP

ACCESSION AP

AP000423

ORGANISM Arabidopsis thaliana*[†]

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; eurosids II; Brassicales; Brassicaceae; Arabidopsis.

ACCESSION AB001684

ORGANISM Chlorella vulgaris[†]

Eukaryota; Viridiplantae; Chlorophyta; Trebouxiophyceae; Chlorellales; Chlorellaceae; Chlorella.

ACCESSION AF137379 L77928

ORGANISM Nephroselmis olivacea

Eukaryota; Viridiplantae; Chlorophyta; Prasinophyceae;

ACCESSIONDQ291132ORGANISMOltmannsiellopsis viridisEukaryota;Viridiplantae;Chlorophyta;Oltmannsiellopsis.	
ACCESSION AP006715 ORGANISM Porphyra yezoensis Eukaryota; Rhodophyta; Bangiophyceae; Bangiales; Bangiaceae; Porphyra.	
ACCESSION U38804 ORGANISM Porphyra purpurea [†] Eukaryota; Rhodophyta; Bangiophyceae; Bangiales; Bangiaceae;	
ACCESSION AY673996 ORGANISM Gracilaria tenuistipitata var. liui [†] Eukaryota; Rhodophyta; Florideophyceae; Gracilariales; Gracilariaceae; Gracilaria.	
ACCESSION AF022186 Z36235 Z70297 ORGANISM Cyanidium caldarium Eukaryota; Rhodophyta; Bangiophyceae; Cyanidiales; Cyanidiacea Cyanidium.	ne;
ACCESSION AB002583 ORGANISM Cyanidioschyzon merolae strain 10D* [†] Eukaryota; Rhodophyta; Bangiophyceae; Cyanidiales; Cyanidiacea Cyanidioschyzon.	ne;
ACCESSION EF067921 ORGANISM Thalassiosira pseudonana * [†] Eukaryota; stramenopiles; Bacillariophyta; Coscinodiscophyceae; Thalassiosirophycidae; Thalassiosirales; Thalassiosiraceae; Thalassiosira.	
ACCESSION Z67753 ORGANISM Odontella sinensi* [†] Eukaryota; stramenopiles; Bacillariophyta; Coscinodiscophyceae; Biddulphiophycidae; Eupodiscales; Eupodiscaceae; Odontella.	
ACCESSION U30821 ORGANISM Cyanophora paradoxa [†] Eukaryota; Glaucocystophyceae; Cyanophoraceae; Cyanophora.	
ACCESSION X70810	

Euglena gracilis[†] ORGANISM Eukaryota; Euglenozoa; Euglenida; Euglenales; Euglena. ACCESSION AY741371 Emiliania huxleyi[†] ORGANISM Eukaryota; Haptophyceae; Isochrysidales; Noelaerhabdaceae; Emiliania. ACCESSION DO630521 L49157 **Stigeoclonium helveticum** ORGANISM Eukaryota; Viridiplantae; Chlorophyta; Chlorophyceae; ACCESSION DQ396875 L43360 ORGANISM Scenedesmus obliguus Eukaryota; Viridiplantae; Chlorophyta; Chlorophyceae; Sphaeropleales; Scenedesmaceae; Scenedesmus. ACCESSION AY835431 L44125 Pseudendoclonium akinetum ORGANISM Eukaryota; Viridiplantae; Chlorophyta; Ulvophyceae; Ulvales; Pseudendoclonium. ACCESSION NC 008289 Ostreococcus tauri ORGANISM Eukaryota; Viridiplantae; Chlorophyta; Prasinophyceae; Mamiellales; Mamiellaceae; Ostreococcus. ACCESSION BK000554 AF396929 ORGANISM Chlamydomonas reinhardtii* Eukaryota; Viridiplantae; Chlorophyta; Chlorophyceae; Chlamydomonadales; Chlamydomonadaceae; Chlamydomonas. ACCESSION NC 005087 Physcomitrella patens subsp. patens ORGANISM Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Bryophyta; Moss Superclass V; Bryopsida; Funariidae; Funariales; Funariaceae; Physcomitrella. ACCESSION AY864816 Phaeodactylum tricornutum*[†] ORGANISM Eukaryota; stramenopiles; Bacillariophyta; Bacillariophyceae; Bacillariophycidae; Naviculales; Phaeodactylaceae; Phaeodactylum. ACCESSION DO851108 **Bigelowiella natans**[†] ORGANISM Eukaryota; Cercozoa; Chlorarachniophyceae; Bigelowiella.

ACCESSION EF508371 ORGANISM **Rhodomonas salina** Eukaryota; Cryptophyta; Cryptomonadaceae; Rhodomonas.

AF041468 AF063017 M76547 U81044 X14171 X14504 X51511 ACCESSION X52158 X52912 X56806 X62348 X62349 Z21976

Guillardia theta[†] ORGANISM

Eukaryota; Cryptophyta; Cryptomonadaceae; Guillardia.

ACCESSION X56695

ORGANISM Ectocarpus siliculosus

Eukaryota; stramenopiles; Phaeophyceae; Ectocarpales; Ectocarpaceae; Ectocarpus.

ACCESSION AB027234 REGION: 4..1086 **Gymnodinium mikimotoi**[†] ORGANISM

in moto. ae; Gymn. Eukaryota; Alveolata; Dinophyceae; Gymnodiniales; Gymnodiniaceae; Gymnodinium.

Supplementary Figure S3

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
G. mikimotoi	1	100.00	88.18	85.45	88.18	86.36	84.55	86.36	86.36	86.36	84.55	84.55	87.27	86.36	84.55	86.36	85.45	86.36	88.18
E. huxleyi	2	0.13	100.00	92.73	95.45	93.64	95.45	91.82	94.55	90.91	91.82	90.91	93.64	94.55	92.73	92.73	92.73	92.73	94.55
T. pseudonana	3	0.16	0.08	100.00	97.27	97.27	92.73	90.00	90.91	91.82	92.73	89.09	91.82	92.73	92.73	90.91	92.73	91.82	90.91
O. sinensis	4	0.13	0.05	0.03	100.00	98.18	91.82	90.91	91.82	92.73	92.73	90.91	92.73	93.64	91.82	91.82	92.73	92.73	92.73
P. tricornutum	5	0.15	0.07	0.03	0.02	100.00	91.82	90.91	91.82	90.91	90.91	90.00	90.91	91.82	90.91	90.00	92.73	90.91	90.91
G. theta	6	0.17	0.05	0.08	0.09	0.09	100.00	93.64	94.55	90.00	90.00	90.00	93.64	94.55	93.64	91.82	93.64	90.91	90.91
P. purpurea	7	0.15	0.09	0.11	0.10	0.10	0.07	100.00	95.45	90.91	87.27	89.09	90.91	91.82	90.91	90.00	91.82	88.18	90.00
G. tenuistipitata var. liui	8	0.15	0.06	0.10	0.09	0.09	0.06	0.05	100.00	90.91	91.82	90.91	92.73	93.64	92.73	91.82	93.64	90.91	92.73
C. merolae	9	0.15	0.10	0.09	0.08	0.10	0.11	0.10	0.10	100.00	90.91	88.18	91.82	92.73	90.91	90.00	90.91	90.00	90.00
B. natans	10	0.17	0.09	0.08	0.08	0.10	0.11	0.14	0.09	0.10	100.00	90.00	94.55	94.55	93.64	94.55	91.82	92.73	92.73
E. gracilis	11	0.17	0.10	0.12	0.10	0.11	0.11	0.12	0.10	0.13	0.11	100.00	92.73	93.64	92.73	91.82	92.73	90.91	90.91
A. thaliana	12	0.14	0.07	0.09	0.08	0.10	0.07	0.10	0.08	0.09	0.06	0.08	100.00	99.09	96.36	98.18	95.45	93.64	91.82
C. vulgaris	13	0.15	0.06	0.08	0.07	0.09	0.06	0.09	0.07	0.08	0.06	0.07	0.01	100.00	97.27	97.27	96.36	94.55	92.73
O. tauri	14	0.17	0.08	0.08	0.09	0.10	0.07	0.10	0.08	0.10	0.07	0.08	0.04	0.03	100.00	95.45	96.36	92.73	91.82
C. reinhardtii	15	0.15	0.08	0.10	0.09	0.11	0.09	0.11	0.09	0.11	0.06	0.09	0.02	0.03	0.05	100.00	95.45	91.82	92.73
C. paradoxa	16	0.16	0.08	0.08	0.08	0.08	0.07	0.09	0.07	0.10	0.09	0.08	0.05	0.04	0.04	0.05	100.00	91.82	90.91
Synechococcus sp.	17	0.15	0.08	0.09	0.08	0.10	0.10	0.13	0.10	0.11	0.08	0.10	0.07	0.06	0.08	0.09	0.09	100.00	95.45
Synechocystis sp.	18	0.13	0.06	0.10	0.08	0.10	0.10	0.11	0.08	0.11	0.08	0.10	0.09	0.08	0.09	0.08	0.10	0.05	100.00

Pairwise comparison chart of D1 (PsbA) amino acid similarities and distances

From a MUSCLE amino acid sequence alignment of a set of PsbA proteins (also see S3) a pairwise comparison of amino acid similarities and distances was inferred. The similarities are shown in % within and above the fields forming the diagonal from top left to bottom right of the chart. The values below the diagonal represent the Jukes-Cantor distances (corrected for multiple substitutions) between the two compared sequences (Jukes and Cantor, 1969). Red background indicates the highest values, blue background indicates the lowest values of the comparison. Highlighted by a black frame are the D1 similarities of all listed species with the three diatoms.

Methods:

All bioinformatic operations were performed using tools provided by the bioinformatic software package CLC Combined Workbench, Version 3.6.1 (CLC bio, Aarhus, Denmark). The pairwise comparison of amino acid sequence similarities/distances for the Q_B pocket was inferred from a MUSCLE alignment (standard settings, maximal 16

iterations, alignment not shown) of the Q_B pocket sequences (amino acid position 198 to 307) from 18 different species (see Fig. S2).

References:

.96. .s 21-32. N Jukes, T. and Cantor, C. (1969). Mammalian Protein Metabolism, chapter Evolution of protein molecules, pages 21-32. New York: Academic Press.

Supplementary File S4

Pigment composition (in mol/100 mol chlorophyll *a* (Chl *a*)) and photosynthetic

properties of the wild-type (WT) and the *psbA* mutants of *Phaeodactylum tricornutum*.

Pigment/Parameter	WT	V219I	F255I	S264A	L275W	
DCMU resistance	/	3	150	3000	500	
Chl a (pg cell ⁻¹)	0.50 ± 0.07	0.57 ± 0.08	0.53 ± 0.17	0.54 ± 0.03	0.57 ± 0.1	
Diadinoxanthin	9.4 ± 2.1	8.2 ± 2.5	8.3 ± 0.4	8.4 ± 1.3	9.0 ± 2.1	
Fucoxanthin	71.3 ± 5.7	74.3 ± 4.9	75.5 ± 2.8	69.4 ± 3.4	76.6 ± 4.0	
Chl c	13.7 ± 0.7	14.7 ± 0.4	14.9 ± 1.3	14.2 ± 0.7	15.9 ± 1.7	
β-carotene	7.7 ± 0.7	6.7 ± 1.0	6.6 ± 0.4	6.5 ± 1.3	6.0 ± 0.1	
Y_{SS} (µg Chl a^{-1})	90 ± 11	103 ± 10	136 ± 7	149 ± 11	47 ± 4	
RC/CS ₀	158 ± 4	194 ± 4	216 ± 2	232 ± 1	n.d.	
PSI:PSII	0.44 ± 0.04	0.46 ± 0.02	0.47 ± 0.03	0.43 ± 0.03	0.56 ± 0.07	
$1/I_{1/2}$ of Y_{SS}	0.58	0.58	0.45	0.35	0.40	
$E_K \ (\mu mol \ photons \ m^{-2} \ s^{-1})$	183 ± 5	189 ± 10	223 ± 20	240 ± 10	231 ± 10	
$J (Q_A Q_B / Q_A Q_B)$	0.56 ± 0.06	0.61 ± 0.07	0.69 ± 0.09	$0.96\pm0.03*$	0.88 ± 0.09	
$I (Q_{A} Q_{B}^{2})$	0.89 ± 0.02	0.82 ± 0.03	0.82 ± 0.04	0.95 ± 0.02	0.81 ± 0.03	
Misses/PSII	0.14 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.23 ± 0.01	n.d.	
F ₀	131 ± 6	141 ± 11	139 ± 7.5	170 ± 9	180 ± 7	
F_v/F_m	0.70 ± 0.02	0.68 ± 0.01	0.69 ± 0.01	0.71 ± 0.03	0.57 ± 0.01	
ΦPSII	0.57 ± 0.06	0.56 ± 0.01	0.49 ± 0.02	0.39 ± 0.02	0.35 ± 0.04	
ET ₀ /CS ₀	182 ± 17	167 ± 4	145 ± 25	89 ± 17	75 ± 28	
μ (d ⁻¹)	2.54 ± 0.2	2.72 ± 0.2	2.44 ± 0.3	2.38 ± 0.2	1.87 ± 0.1	

Y_{SS} is the concentration of active PSII reaction centers per Chl *a* (Lavaud et al. 2002a); RC/CS₀ is the number of active PSII reaction center per PSII cross-section; 1/I_{1/2} of Y_{SS} is a measurement of the PSII light-harvesting antenna size (Lavaud et al. 2002a); E_K is the light intensity for saturation of photosynthesis; the DCMU resistance factor was calculated from the IC₅₀ PSII fluorescence reactivity to DCMU; J (* J phase is delayed to 9.7 ± 2.5 ms in S264A) and I are the fluorescence levels of the J and I phases measured at 2 ms and 30 ms, respectively (values normalized to the P peak = 1, see Fig. 4A, P corresponds to the concentration of Q_A²Q_B²⁻ and PQH₂); F₀ is the minimal level of fluorescence of dark-adapted cells; F_v/F_m is the maximum photosynthetic efficiency of PSII, Φ PSII ((Fm'-F')/ Fm') is the effective PSII quantum yield for photochemistry measured at 50-100 µmol photons m⁻² s⁻¹ (an irradiance for which there is no NPQ); ET₀/CS₀ is the steady-state electron transport in a PSII cross-section; µ is the growth rate. All measurements were performed on cells grown at 50 µmol photons m⁻² s⁻¹, which was low enough to prevent the de-epoxidation of diadinoxanthin into diatoxanthin. n. d., not determined. Values are average ± SD of three to four measurements. See the text for other details.

Methods:

DCMU resistance was evaluated measuring the inhibition of the PSII activity *vs* increasing DCMU concentrations using a self-made fluorometer (Parésys et al. 2005). The measure of inhibition is the increase in the amplitude of the I-45 ms peak illustrating the accumulation of Q_A^- due to the electron Q_A - Q_B transfer being blocked by DCMU (see Fig. 4B). This way, the IC₅₀, the DCMU concentration at which the PSII activity is inhibited by 50% was measured and compared between WT and mutants to evaluate a resistance factor.

Standard fluorescence nomenclature was used. F_0 and F_m are defined as the minimum PSII fluorescence yield of dark-adapted cells and the maximum PSII fluorescence yield reached

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in such cells during a saturating pulse of white light, respectively. The PSII quantum yield for photochemistry is the ratio F_v/F_m where F_v is the variable part of the fluorescence emission and is equal to F_m - F_0 . The photochemical quenching was $qP = (F_m - F')/(F_m' - F_0')$, where F' is the steady-state level of fluorescence emission reached after few minutes of illumination. 1-qP is a parameter to estimate the fraction of reduced Q_A (Büchel and Wilhelm 1993, Niyogi et al. 1998). A classic OIJP analysis was also performed (Strasser et al. 1995, Force et al. 2003) allowing to extract the following data: time to reach the J phase ($Q_A Q_B^{-7} Q_A^{-1} Q_B^{-1}$ state), time to reach the I phase ($Q_A Q_B^{-2}$ state), and the parameters RC/CS₀ and ET₀/CS₀ (Force et al. 2003).

For the control measurement of the relative O₂ yield produced per flash during a sequence of single-turnover saturating flashes (O₂ sequences), cells were first dark-adapted for 20 min and then deposited on the electrode. The cells were allowed to settle on the electrode for 7 min in darkness before measurement. The steady-state O_2 yield per flash (Y_{SS}) was attained for the last four flashes of a sequence of 20 flashes when the classical four-step oscillations due to the S-state cycle of the PSII RC O₂-evolving complex (Kok et al. 1970); see Fig. 4 for a classic recording) were fully damped. The oscillation is damped after a few cycles to a constant steady-state yield, Y_{SS}, due to loss of synchrony caused mainly by a nonzero probability of 'misses' (reflecting the number of PSII RCs unable to perform a charge separation during a flash, Lavorel 1976). Y_{SS} was used to evaluate the number of O₂ producing PSII RCs relative to Chl a (see Lavaud et al. 2002). The miss probability per PS II was calculated from the Lavorel matrix (Lavorel 1976). For the saturation curves of Y_{SS}, the intensity of the flashes was varied with neutral density filters. A new dark-adapted sample was used for each flash intensity measurement. $1/I_{1/2}$ of Y_{SS} , the reciprocal of the half-saturating flash intensity of flash O₂ evolution saturation curves was used as a measure of the PSII antenna size (Lavaud et al. 2002).

 E_K , the irradiance for saturation of photosynthetic O_2 emission was estimated from P/E curves.

References:

Büchel, C. & Wilhelm, C. 1993. *In vivo* analysis of slow chlorophyll fluorescence induction kinetics in algae: progress, problems and perspectives. *Photochem. Photobiol.* 58:137-48
Force, L., Critchley, C. & van Rensen, J. J. S. 2003. New fluorescence parameters for monitoring photosynthesis in plants. 1. The effect of illumination on the fluorescence parameters of the JIP-test. *Photosynth. Res.* 78:17-33

Kok, B., Forbush, B. & McGloin, M. 1970. Cooperation of charges in photosynthetic O₂ evolution-I. A linear four step mechanism. *Photochem. Photobiol.* 11:457-75

Lavaud, J., van Gorkom, H. & Etienne, A.-L. 2002. Photosystem II electron transfer cycle and chlororespiration in planktonic diatoms. *Photosynth. Res.* 74:51-59

Lavorel, J. 1976. Matrix analysis of the oxygen evolving system of photosynthesis. *J. Theor. Biol.* 57:171-85

Niyogi, K. K., Grossman, A. R. & Bjorkman, O. 1998. *Arabidopsis* mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. *Plant Cell* 10:1121-34

Parésys, G., Rigart, C., Rousseau, B., Wong, A. W. M., Fan, F., Barbier, J.-P. & Lavaud, J. 2005. Quantitative and qualitative evaluation of phytoplankton populations by trichromatic chlorophyll fluorescence excitation with special focus on cyanobacteria. *Water Res.* 39:911-21

Strasser, R. J., Srivasatava, A. & Govindjee 1995. Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. *Photochem. Photobiol.* 61:32-4

Supplementary File S5

Growth curves of the wild-type (WT) and the four *psbA* V219I / F255I / S264A / L275W mutants of *P. tricornutum* cells.

Growth curves were performed under a light regime of 50 μ mol photons m⁻² s⁻¹/16:8 h light:dark. The cells were sampled each day 3 h after initiating the light period. Values are average \pm SD of three to four measurements.





Figure S1 201x159mm (600 x 600 DPI)



DE helix

E helix

Figure S2 203x210mm (600 x 600 DPI)



		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
G. mikimotoi	1	100.00	88.18	85.45	88.18	86.36	84.55	86.36	86.36	86.36	84.55	84.55	87.27	86.36	84.55	86.36	85.45	86.36	88.18
E. huxleyi	2	0.13	100.00	92.73	95.45	93.64	95.45	91.82	94.55	90.91	91.82	90.91	93.64	94.55	92.73	92.73	92.73	92.73	94.55
T. pseudonana	3	0.16	0.08	100.00	97.27	97.27	92.73	90.00	90.91	91.82	92.73	89.09	91.82	92.73	92.73	90.91	92.73	91.82	90.91
O. sinensis	4	0.13	0.05	0.03	100.00	98.18	91.82	90.91	91.82	92.73	92.73	90.91	92.73	93.64	91.82	91.82	92.73	92.73	92.73
P. tricornutum	5	0.15	0.07	0.03	0.02	100.00	91.82	90.91	91.82	90.91	90.91	90.00	90.91	91.82	90.91	90.00	92.73	90.91	90.91
G. theta	6	0.17	0.05	0.08	0.09	0.09	100.00	93.64	94.55	90.00	90.00	90.00	93.64	94.55	93.64	91.82	93.64	90.91	90.91
P. purpurea	7	0.15	0.09	0.11	0.10	0.10	0.07	100.00	95.45	90.91	87.27	89.09	90.91	91.82	90.91	90.00	91.82	88.18	90.00
G. tenuistipitata var. liui	8	0.15	0.06	0.10	0.09	0.09	0.06	0.05	100.00	90.91	91.82	90.91	92.73	93.64	92.73	91.82	93.64	90.91	92.73
C. merolae	9	0.15	0.10	0.09	0.08	0.10	0.11	0.10	0.10	100.00	90.91	88.18	91.82	92.73	90.91	90.00	90.91	90.00	90.00
B. natans	10	0.17	0.09	0.08	0.08	0.10	0.11	0.14	0.09	0.10	100.00	90.00	94.55	94.55	93.64	94.55	91.82	92.73	92.73
E. gracilis	11	0.17	0.10	0.12	0.10	0.11	0.11	0.12	0.10	0.13	0.11	100.00	92.73	93.64	92.73	91.82	92.73	90.91	90.91
A. thaliana	12	0.14	0.07	0.09	0.08	0.10	0.07	0.10	0.08	0.09	0.06	0.08	100.00	99.09	96.36	98.18	95.45	93.64	91.82
C. vulgaris	13	0.15	0.06	0.08	0.07	0.09	0.06	0.09	0.07	0.08	0.06	0.07	0.01	100.00	97.27	97.27	96.36	94.55	92.73
O. tauri	14	0.17	0.08	0.08	0.09	0.10	0.07	0.10	0.08	0.10	0.07	0.08	0.04	0.03	100.00	95.45	96.36	92.73	91.82
C. reinhardtii	15	0.15	0.08	0.10	0.09	0.11	0.09	0.11	0.09	0.11	0.06	0.09	0.02	0.03	0.05	100.00	95.45	91.82	92.73
C. paradoxa	16	0.16	0.08	0.08	0.08	0.08	0.07	0.09	0.07	0.10	0.09	0.08	0.05	0.04	0.04	0.05	100.00	91.82	90.91
Synechococcus sp.	17	0.15	0.08	0.09	0.08	0.10	0.10	0.13	0.10	0.11	0.08	0.10	0.07	0.06	0.08	0.09	0.09	100.00	95.45
Synechocystis sp.	18	0.13	0.06	0.10	0.08	0.10	0.10	0.11	0.08	0.11	0.08	0.10	0.09	0.08	0.09	0.08	0.10	0.05	100.00

Figure .78mm (600 .



Figure S5 121x118mm (600 x 600 DPI)