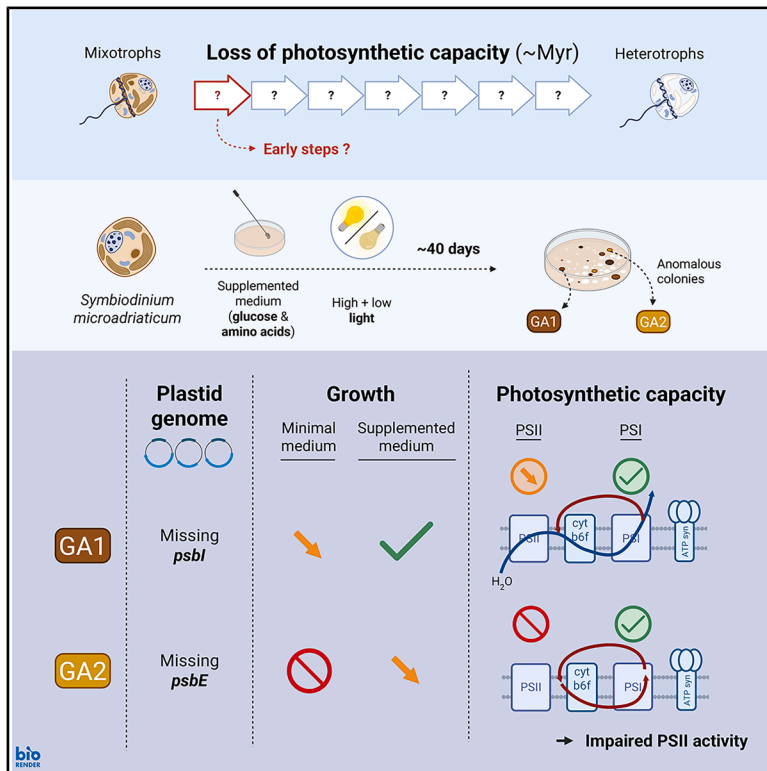


Current Biology

Spontaneous evolution of heterotrophy in cultured dinoflagellate algae

Graphical abstract



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In brief

Barbrook et al. show the dinoflagellate alga *Symbiodinium microadriaticum* can spontaneously lose photoautotrophy by losing one of the chloroplast subgenomic minicircles and photosystem II activity. Photosystem I and cyclic electron flow persist. This novel model system for directly observing the transition to heterotrophy may also help us understand dinoflagellate cellular evolution.

Highlights

- A dinoflagellate alga (*Symbiodinium*) can readily lose photoautotrophy in the lab
- This is due to the loss of a minicircle from the fragmented algal chloroplast genome
- Missing minicircles encode photosystem II subunits (PsbE/I), resulting in PSII loss
- Cyclic electron flow around PSI is maintained, possibly supporting ATP synthesis

Article

Spontaneous evolution of heterotrophy in cultured dinoflagellate algae

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SUMMARY

Acquisition of chloroplasts by an early eukaryote and their subsequent spread across eukaryotes is well understood. However, evolution can be undone, and many important lineages (especially in the Apicomplexa) have lost photosynthesis, returning to heterotrophy. Spontaneous transition to heterotrophy remains difficult to study, as most examples happened on an evolutionary timescale. Here, we describe frequent transition to heterotrophy in a dinoflagellate alga under laboratory conditions. Dinoflagellates, close relatives of Apicomplexa, have an unusual chloroplast genome, highly reduced and fragmented into subgenomic “minicircles,” each typically with a single gene encoding a component of the photosynthetic electron transfer chain. This extraordinary genome organization may predispose to loss of gene(s) essential for photosynthesis. We found that growing the dinoflagellate *Symbiodinium microadriaticum* (a strain able to form symbioses with corals and other Cnidaria) on medium supplemented with glucose and amino acids allowed the ready isolation of multiple strains with spontaneous partial or complete loss of photosynthetic growth, resulting from the loss of a chloroplast subgenomic minicircle. Different strains showed independent loss of a minicircle encoding one of the photosystem II (PSII) components PsbE or PsbI. Spectroscopic analysis confirmed loss/impairment of PSII and retention of photosystem I (PSI) and cyclic electron flow (CEF), probably providing ATP. This system should allow the study of the spontaneous transition to heterotrophy and the role of intermediate stages in maintaining CEF. The ready loss of photosynthesis in dinoflagellates resulting from their chloroplast genome organization may explain why dinoflagellates lost or replaced chloroplasts several times during evolution.

INTRODUCTION

How photosynthetic chloroplasts originated by endosymbiosis has been studied in great detail.^{1,2} The loss of photosynthesis has occurred many times across the eukaryotic tree of life, in multicellular organisms as well as unicellular ones, but the process of loss is less well understood than chloroplast gain.³ In some cases, such as the plant *Epifagus virginiana* or single-celled organisms such as *Plasmodium falciparum*, the chrysophyte alga *Spumella* sp., and *Euglena (Astasia) longa*, a remnant, non-photosynthetic chloroplast is retained with a reduced genome that lacks, or has pseudogenes for, most or all of the genes for the light and dark reactions of photosynthesis.^{4–7} In some other organisms, such as the “green” alga *Polytomella* spp. and the land plant *Rafflesia lagascae*, the chloroplast genome has been lost completely, although a remnant chloroplast organelle is retained.⁸ Many of the other biochemical

functions of plastids are retained in the remnant organelles, although closely related lineages show differences in which plastid-targeted proteins are retained.⁹ It is believed to be beneficial to retain a subcellular compartment to maintain different metabolite concentrations from the rest of the cell or to contain reaction intermediates. Nevertheless, some organisms, such as the parasitic dinoflagellate *Hematodinium* sp. and the parasite *Cryptosporidium hominis*, seem to have lost the remnant chloroplast completely.^{10,11} There is therefore a very wide range of organisms that have lost photosynthesis and chloroplast function to different extents. However, these non-photosynthetic organisms have been separated from their closest photosynthetic relatives for many millions of years,¹² which makes it difficult to identify the changes directly associated with the transition to obligate heterotrophy. There are instances of non-photosynthetic strains of photosynthetic organisms being generated on a laboratory timescale, but they generally involve the use of

non-physiological treatments or mutagenesis. In other cases, the strains have been in culture for a considerable time, the exact parental strain is not clear, and large-scale ultrastructural rearrangements have occurred.^{5,13–15}

Understanding the early stages in the spontaneous transition to heterotrophy is therefore very difficult with the organisms available. We reasoned that peridinin-containing dinoflagellate algae might be prone to spontaneous loss of photoautotrophy, given the fragmented nature of their chloroplast genome. Instead of having the chloroplast genes present on a single 120–200 kb DNA molecule, the dinoflagellate chloroplast genome is made up of multiple plasmid-like minicircles, typically 2–5 kbp, which are located in the chloroplast. Each minicircle carries one or a few genes as well as a “core” region containing the origin of replication. The non-coding regions of the minicircles do not show any obvious sequence similarity to conventional chloroplast genomes. Fewer than 20 genes in total are retained in the chloroplast on these minicircles. These genes encode proteins involved in photosynthesis, rRNAs, and a small number of tRNAs.^{16–18} The copy number of the minicircles can be as low as a few per cell, depending on the growth conditions.^{19,20} We argued that the dependence of the photosynthetic machinery on multiple minicircles, some of which are at low copy number, might lead to a frequent generation of strains lacking one or more components of the photosynthetic machinery as a result of loss of a minicircle. We chose to look for spontaneous loss of photoautotrophy in the dinoflagellate *Symbiodinium*, which is known to be capable in principle of photoautotrophic, mixotrophic, or heterotrophic growth²¹ and can be grown on solid medium, which should allow non-photosynthetic mutant strains to be recovered. The strain can also form symbioses with corals. We found that mutants impaired in, or completely lacking, photosynthetic growth could be readily obtained. A number of these were found to have lost a minicircle encoding a photosystem II (PSII) component. These components were PsbE, a subunit of the cytochrome *b*₅₅₉ component of PSII that is essential for PSII function, and PsbI, which is involved in PSII assembly and/or stability and whose loss leads to partial loss of PSII function.²² Biophysical analysis showed that these strains retained photosystem I (PSI) function and had lost some or all of PSII function. Retention of PSI presumably allows some benefit from cyclic electron flow (CEF) while not providing the NADPH required for carbon fixation. This phenomenon offers a novel system for studying the spontaneous loss of photosynthesis on a lab timescale and may provide an explanation for the frequency with which dinoflagellates have lost photosynthesis or replaced their chloroplasts with chloroplasts from elsewhere.²³

RESULTS

Minicircle-deficient mutant strains can be readily isolated

Symbiodinium microadriaticum cells from a liquid culture were spread onto plates containing f/2 solid medium and incubated at 25°C with illumination at 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ to form a continuous patch. Cells from this patch were respread onto f/2 solid medium plates supplemented with glucose and casamino acids (amino acids derived from casein hydrolysis) and incubated under higher light of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 25°C.

After 10 days, the plates were moved to lower light conditions (10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ photons at 25°C) and allowed to continue growing. After 28 days, many of the resulting cells had bleached and appeared white. These bleached cells were incapable of further growth even on supplemented medium. However, within this background of bleached cells, colonies of pigmented cells were observed. These colonies showed variation in colony size, pigmentation, and morphology, as shown in Figure 1A.

To determine what had caused the change in phenotype, we picked a single small colony showing lighter pigmentation than the majority for analysis. This colony, subsequently designated GA1 (glucose and amino acid 1), was first streaked out on supplemented f/2 agar (Figure 1B) and used to establish liquid cultures that were incubated for 30 days under moderate light (40 $\mu\text{mol photons m}^{-2} \text{s}^{-2}$) with or without glucose supplementation (Figures 1C and 1D). The GA1 strain showed very little growth in f/2 medium without glucose supplementation. By contrast, its growth rate was similar to wild-type (WT) cells in medium supplemented with glucose, suggesting at least a partial loss of photosynthetic capacity. To test the hypothesis that loss of photosynthesis might result from loss of a chloroplast DNA minicircle, we analyzed DNA from strain GA1 by PCR using inward-facing primer pairs for the coding region of each minicircle and, for confirmation, outward-facing primers for the rest of each minicircle (Figure 2). The results showed very similar amplification products (in size of product and intensity of band) between the WT and GA1 for all minicircles except for the one containing *psbI*, for which we were repeatedly unable to obtain products using inward or outward PCR. To test if other strains showing unusual morphology or pigmentation had lost minicircles, six more were analyzed by PCR (Figure S1). One of these also appeared to be lacking the *psbI* minicircle, based on the failure to amplify it by PCR, and two appeared to be lacking the *psbE* minicircle. One of the strains missing *psbE*, designated GA2 (Figure 1B), was chosen for further analysis. Three of the additional strains appeared to contain a full set of minicircles, although mutations in minicircles could not be excluded. To confirm the absence of the minicircles from GA1 and GA2, we isolated total DNA from GA1, GA2, and WT strains and carried out next generation sequencing genome analysis. The results confirmed the absence of the *psbI* minicircle from GA1 and the absence of *psbE* from GA2 (Figure S2).

Photosynthetic activity in minicircle-deficient strains

We conducted growth tests on the strains across a range of light intensities (Figure 1E). The initial results indicated that the mutant strains exhibited reduced growth compared with the WT (GA1), or no growth at all (GA2), in the absence of supplements. However, in the presence of glucose and amino acids, both mutant strains demonstrated improved growth at low light intensities compared with growth in complete darkness, suggesting some benefit from photosynthesis. In addition, the GA2 strain appeared to be more sensitive to high light intensities than the other strains.

We characterized the photosynthetic function of the GA1 and GA2 strains (Figures 3, 4, and 5; Table 1). The maximum quantum yield of PSII in the WT was approximately 0.55, whereas it reached only 0.25 in GA1 and was zero in GA2 (Figure 3A). These

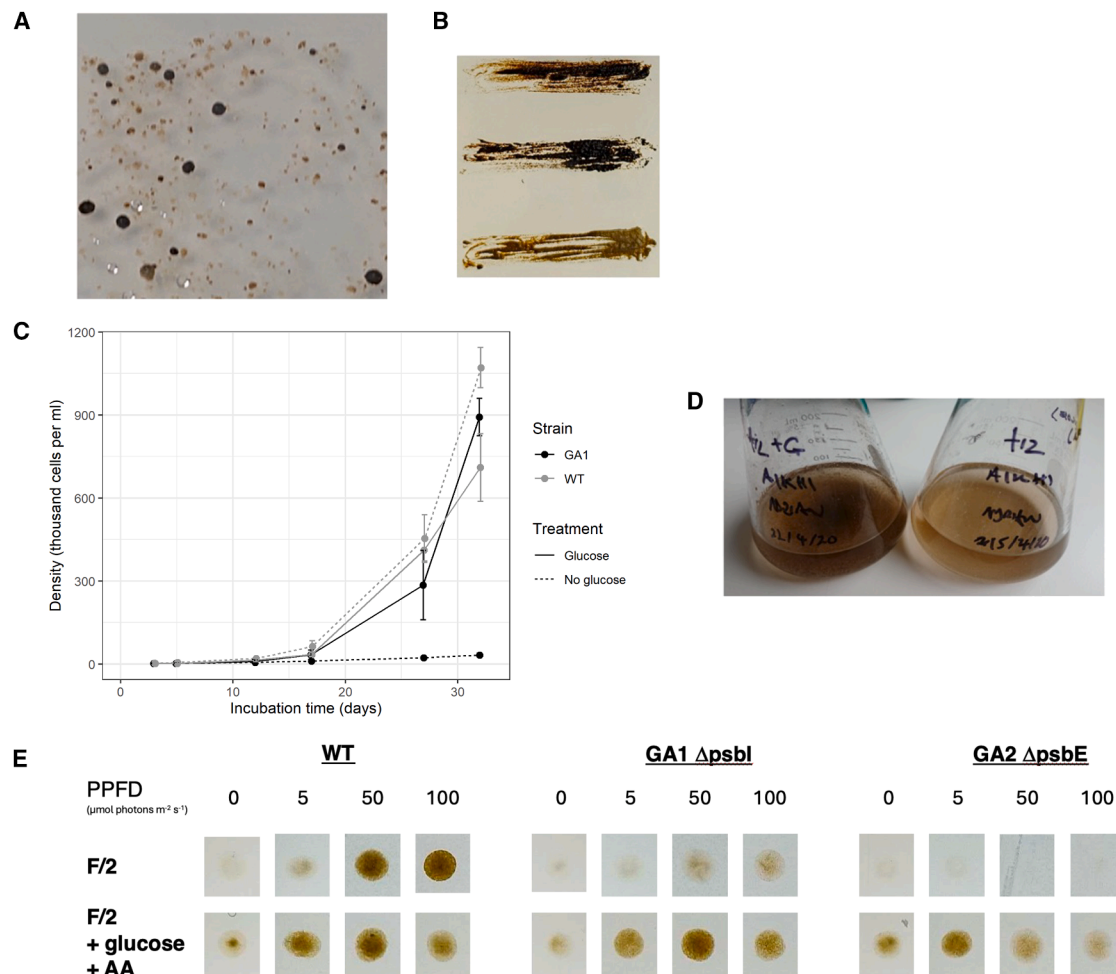


Figure 1. Mutant lines of *S. microadriaticum*

(A) The variation in colony morphology seen when cells were incubated on a rich medium in the light. There is a poorly defined background of pale or bleached material that does not form clear colonies capable of growth. Within this, individual colonies can be discerned, with a range of sizes and pigmentation.

(B) The WT (upper), GA1 (middle), and GA2 (lower) strains.

(C) Growth of GA1 and WT cells under moderate light (40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) over 30 days, with or without glucose. Error bars show standard deviation for $n = 3$ biological replicates.

(D) Cultures of GA1 after 3 months of incubation (left, with glucose; right, without glucose).

(E) The growth of GA1 and GA2 spotted onto f/2 agar (upper panel) or f/2 agar with supplement (lower) and incubated for 2 weeks at the photon flux densities indicated.

results indicate that PSII is partially functional in the absence of PsbI (GA1), while PSII is non-functional in the PsbE-null mutant strain (GA2). This conclusion was further supported by measurements of the relative electron transport rate through PSII (rETR-PSII), which remained null in GA2 even under increasing light intensities (Figure 3B). In GA1, the maximum rETR-PSII reached levels comparable to the WT when short light periods (3 s) were applied (Figure 3B). However, with longer illumination periods (3 min), the maximum rETR-PSII in GA1 was lower than in the WT, suggesting that the absence of PsbI in GA1 reduced efficient electron transfer in PSII under continuous high light (Figure 3C). We also assessed the relative amounts of active photosystems compared with the amount of active PSII in the WT by measuring the A phase of the electrochromic shift (Figure 4). These data confirmed that the amount of active PSII

is reduced in GA1, while GA2 completely lacks active PSII. This analysis also revealed that the amount of active PSI is similar between both PSII-impaired mutants and the WT.

Measurements of absorption at 705 nm (Figure 5A) confirmed that the amount of P700, the primary electron donor in PSI reaction centers, was similar between mutant and WT strains. However, these measurements also showed that the quantum yield of PSI (Y(I)) was significantly lower in GA1 and GA2 compared with the WT (Figures 5B and 5C). In addition, the limitation of PSI at the donor side (Y(ND)) in the absence of PSII inhibitors was most pronounced in GA2 (Figures 5B and 5D). This is consistent with the fact that PSI cannot receive electrons from PSII. GA1, with partial PSII functionality in the absence of PsbI, exhibited an intermediate phenotype between GA2 and the WT. There was little evidence of acceptor-side limitation (Y[NA])

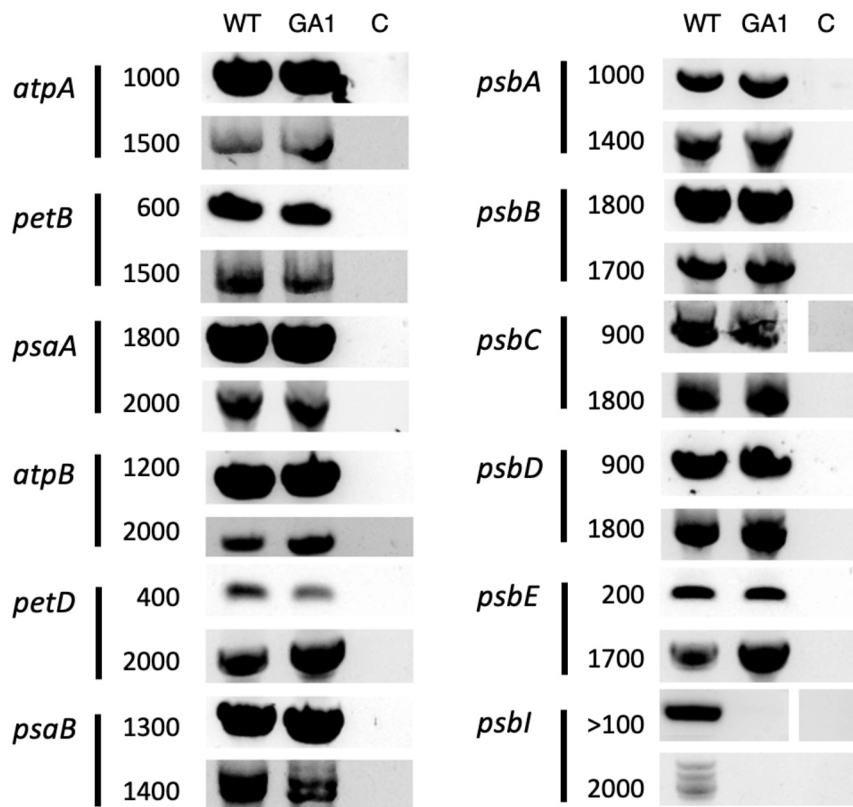


Figure 2. PCR analysis of the chloroplast DNA minicircles in the mutant GA1

DNA from WT and GA1 cells was used for PCR with inward-facing primers for minicircle genes (upper panel for each gene) and outward-facing primers (lower panel). The numbers by each panel indicate the expected size of the PCR product, which was consistent with observed values. C is a control PCR without a template. The order of lanes in the *psbC* and *psbI* inward PCR panels was edited, indicated by the white spacer, to be consistent with the other panels. See also [Figures S1](#) and [S2](#).

([Figure 4](#)), strongly suggests that CEF plays a major role in maintaining PSI activity in the mutant strains, especially GA2.

The observed effects of *PsbI* and *PsbE* loss on PSII function are consistent with those seen in other organisms following targeted mutagenesis. Loss of *PsbI* is generally associated with defects in assembly and/or stability of PSII.²⁶ We observed that in *Symbiodinium*, *PsbI* loss resulted in very slow photoautotrophic growth, a reduction of about 50% in PSII function, and increased susceptibility to photodamage. This is consistent

of PSI in any of the strains, including the WT ([Figure 5B](#)). Moreover, the addition of both the PSII inhibitors DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and hydroxylamine had less impact on PSI activity in the mutants than in the WT ([Figures 5B](#) and [5C](#)), consistent with the reduced or absent PSII function in GA1 and GA2. As expected, the donor-side limitation showed an opposite trend when PSII inhibitors were applied, remaining unchanged in GA2, slightly increasing in GA1, and significantly increasing in the WT ([Figures 5B](#) and [5D](#)). Interestingly, even in the presence of PSII inhibitors, PSI activity in the mutants was slightly higher than in the WT ([Figure 5B](#)).

DISCUSSION

Phenotype of the mutants

The results are summarized in [Table 1](#). Overall, these results indicate a substantial loss of PSII function in the absence of *PsbI*, with the remaining PSII highly susceptible to photodamage. In the *PsbE*-deficient mutant strain, there is a complete loss of PSII function. The partial or complete loss of PSII function is associated with an increased PSII-independent PSI activity in both mutant strains. This PSII-independent PSI activity indicates the occurrence of CEF around PSI, which would generate ATP without producing reducing equivalents and is known to occur in dinoflagellates.^{24,25} In GA2, where PSII is entirely non-functional and no acceptor-side limitation is observed, CEF must account for nearly all of the PSI activity. The fact that significant PSI function persists in the presence of PSII inhibitors, combined with the higher relative abundance of PSI compared with PSII

with observations that *PsbI* knockout strains of *Chlamydomonas reinhardtii* show approximately 10%–20% of WT PSII function and can grow photoautotrophically (but more slowly than WT) under low light but are sensitive to photodamage.²⁷ A mutant of the cyanobacterium *Synechocystis* sp. PCC6803 lacking *PsbI* also shows photosensitivity and decreased PSII activity.²⁸ (The fact that the *Symbiodinium* mutant retains some PSII function and slow photoautotrophic growth, as with *PsbI* loss in other organisms, indicates that the loss of the *PsbI* minicircle is the primary defect rather than being a subsequent mutation following loss of photosynthetic function as a result of some other unknown mutation.) In contrast to the situation with *PsbI*, loss of *PsbE* (a subunit of cytochrome *b₅₅₉* that is essential for PSII function) in *C. reinhardtii* leads to loss of photoautotrophic growth and PSII function,²⁹ as seen here with *Symbiodinium*.

Evolutionary implications

Of seven strains showing altered morphology or pigmentation that were analyzed, four showed loss of a chloroplast DNA minicircle, all encoding a gene for a PSII protein (two strains had lost the *PsbE* minicircle and two had lost the *PsbI* minicircle). There are 14 minicircles in the chloroplast genome of *S. microadriaticum*, encoding components of the photosynthetic machinery or rRNA. Six of these encode components of PSII.³⁰ It will be interesting to determine which other minicircles, if any, are absent from similar mutants. Nevertheless, if all minicircles were equally likely to be lost, the probability that four randomly selected minicircle-deficient lines had all lost a minicircle encoding a PSII component would be approximately 0.03. Our results therefore suggest that there is a bias in favor of recovering lines

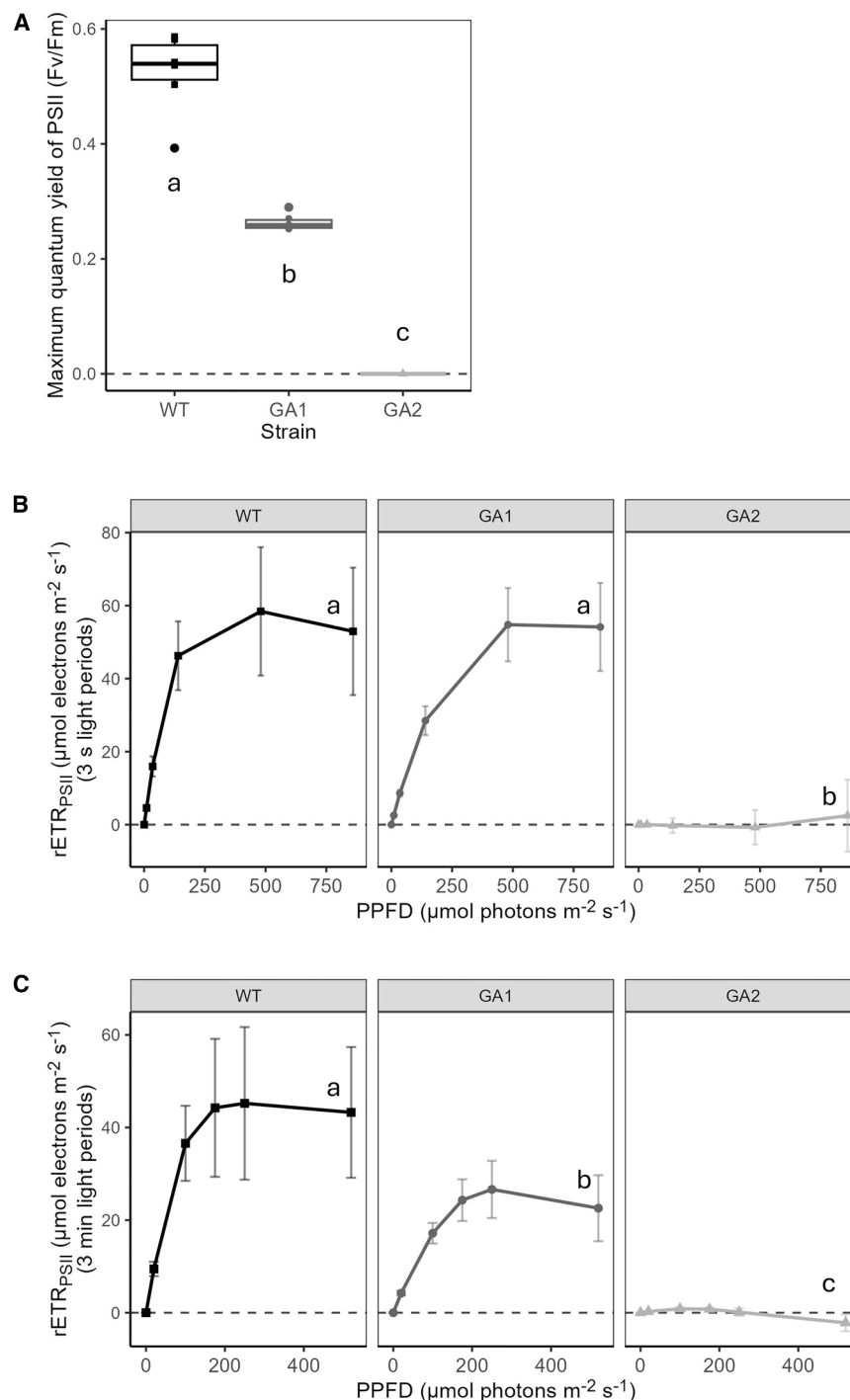


Figure 3. PSII function in the mutants

(A) The ratio F_v/F_m as a measure of the maximum quantum yield of PSII for WT and mutant strains. Different letters indicate values that are statistically significantly different ($p < 0.05$ multiple t test with Benjamini-Hochberg adjustment, $n = 6$). The values of F_v/F_m in GA2 were negative (from -0.044 to -0.067) and therefore judged as biased and set to 0.

(B and C) Relative electron transport rate through PSII ($rETR_{PSII}$) as a function of photosynthetic photon flux density (PPFD) using light periods of 3 s (B) or 3 min (C). Note that the WT and GA1 display similar maximum $rETR_{PSII}$ with 3 s light periods, but the GA1 values are significantly lower than the WT with 3 min light periods, suggesting photodamage in GA1. Different letters (a, b, and c) indicate maximum values of $rETR_{PSII}$ (at 860 or 520 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 3 s or 3 min light periods, respectively) that are statistically significantly different (a from b, etc., $p < 0.05$, multiple t test for 3 s and Wilcoxon test for 3 min light periods, respectively (with Benjamini-Hochberg adjustment, normality checked using Shapiro-Wilk test), $n = 6$ in both cases, with separate tests for measurements at 3 s and 3 min light periods). Error bars (in B and C) represent standard deviation (SD).

PSI. In an evolutionary context, we predict that an organism moving from a mixotrophic lifestyle (a common lifestyle in marine protists, for example³²) to a heterotrophic one would initially lose PSII function. This would allow the benefit of ATP generation from CEF as an intermediate stage. Once an adequate pathway for ATP production via heterotrophy alone had been established, CEF could safely be dispensed with, and there would be no selective pressure for it to remain in illuminated environments. Consistent with this prediction, there are a number of instances of organisms that have lost PSII but retained PSI. The cyanobacterium *Candidatus Atelocyanobacterium thalassa* (also known as UCYN-A) forms a symbiotic association with a prymnesiophyte alga. It is believed to provide the latter with fixed nitrogen in return for fixed carbon and has been described as a “nitroplast.”³³ The cyano-

bacterium probably retains the capacity for CEF and ATP generation but has lost PSII function.³⁴ The long-term, haptophyte-derived kleptoplast of the Antarctic Ross Sea dinoflagellate also appears to have retained the capacity for cyclic, but not linear, electron transfer.³⁵ A particularly remarkable example of PSII loss comes from the warnowiid dinoflagellates, which have an eye-like organelle of chloroplast origin. In at least two independent instances in the warnowiids, PSII has been lost, but PSI has been retained.³⁶

that have lost PSII rather than any of the other photosynthetic complexes. In *Chlamydomonas reinhardtii*, PSII activity in the absence of a functional PSI causes photodamage, given the lack of an adequate electron sink.³¹ It therefore seems likely that PSII-deficient mutants were recovered preferentially here, as lines lacking PSI (and not PSII) would have reduced viability because of photodamage. Our results indicate that CEF occurs in the PSII mutants, which would allow some benefit from light-dependent production of ATP or other beneficial functions of

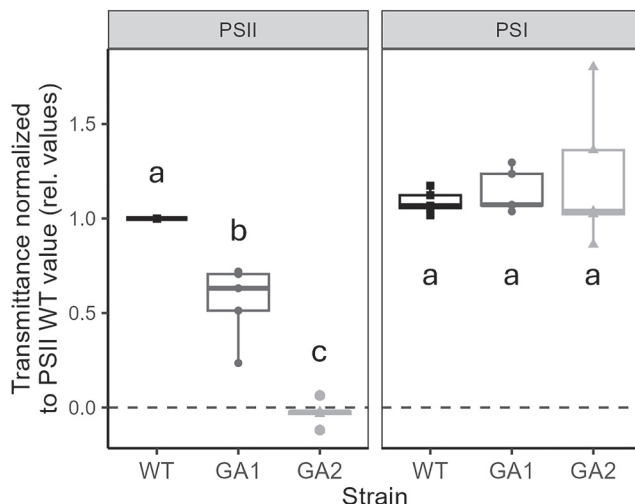


Figure 4. Relative amounts of active photosystems in the mutants compared with active PSII amount in the WT

The figure shows the absorbance values corresponding to the A phase of the electrochromic shift signal (ECS) in response to a single-turnover laser flash, normalized to the chlorophyll concentration of the sample and to the value of active PSII in the WT of the same replicate. Note that in the WT strain, as previously observed, the relative amount of both photosystems is similar, indicating that there is as much active PSII as there is active PSI, and therefore suggesting linear electron flow. This balance is not observed in GA1, where the relative amount of PSI is approximately twice as high as that of PSII. In GA2, the relative amount of PSI is the only one observed, while it is null for PSII. Different letters indicate values that are statistically significantly different (a from b, etc., $p < 0.05$, Tukey's test [normality checked using Shapiro-Wilk test], $n = 6$).

The frequent observation of *Symbiodinium* colonies with abnormal pigmentation, a significant proportion of which have lost one of the chloroplast DNA minicircles, confirms our hypothesis that it would be easy for dinoflagellates to lose photosynthetic capacity by loss of one or more minicircles. The fact that this has not, to our knowledge, been described before in lab cultures may reflect the limited number of dinoflagellate strains that can be conveniently grown as isolated colonies on solid media. In some field isolates of the dinoflagellate *Esotrodinium*, heterotrophy appears to be associated with loss of the *psbA* gene, which is usually present on a chloroplast minicircle,³⁷ and this may be another instance of the phenomenon we describe here. The ease of loss of a minicircle is consistent with the small number of copies of each minicircle in dinoflagellate chloroplasts. This varies with growth conditions, and for some minicircles may be as low as around four copies per cell.²⁰ So unless there are accurate mechanisms to ensure partitioning of all minicircles to daughter cells on division, loss on a lab timescale is not surprising.

The spontaneous loss we describe here offers a system for easily obtaining photosynthesis-deficient strains in a lab timescale. Based on this, we hypothesize that initial loss of PSII, with retention of PSI, is a common early step in the transition to heterotrophy for dinoflagellates in nature. In the longer term, it will be interesting to look in detail at subsequent changes in these strains over time, such as changes in the transcript or proteomic profiles in response to PSII loss or loss of further minicircles. Such studies will provide further insights into the

subsequent stages of loss of photosynthesis. These might include loss of PSI, as noted in the examples above of organisms that have been heterotrophic for a long time. We also expect that many more examples will become apparent of organisms that have lost PSII but retained PSI and CEF.

One of the most remarkable and puzzling features of dinoflagellates is the large fraction of species that are obligately heterotrophic, perhaps as many as 50%.³⁸ In several taxa, chloroplasts have been replaced by a chloroplast obtained from another source,²³ or even a whole photosynthetic organism has been acquired as a new symbiont.³⁹ Loss of a minicircle would impose a very strong selective pressure on a dinoflagellate cell either to develop (or enhance) heterotrophy or to acquire a new chloroplast from a different source. The demonstration here of the frequent spontaneous loss of minicircles from the fragmented dinoflagellate chloroplast genome provides a ready explanation for the remarkable evolutionary propensity of this important algal group to substitute its chloroplasts or become obligately heterotrophic.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Christopher Howe (ch26@cam.ac.uk).

Materials availability

Strains described in this study will be made available on request, subject to international shipping regulations for biological materials, and with reasonable compensation by the requestor for processing and shipping. We may require a completed materials transfer agreement.

Data and code availability

- Sequence data are available at <https://www.ncbi.nlm.nih.gov/bioproject/1330361> and are publicly available as of the date of publication. All raw data supporting the photosynthetic measurements reported in this study are available on Zenodo: <http://www.doi.org/10.5281/zenodo.17360039>.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

All authors contributed to the experimental design and the drafting of the manuscript. A.C.B., E.R., and F.B. generated the data. The graphical abstract was created by E.R. in BioRender.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE

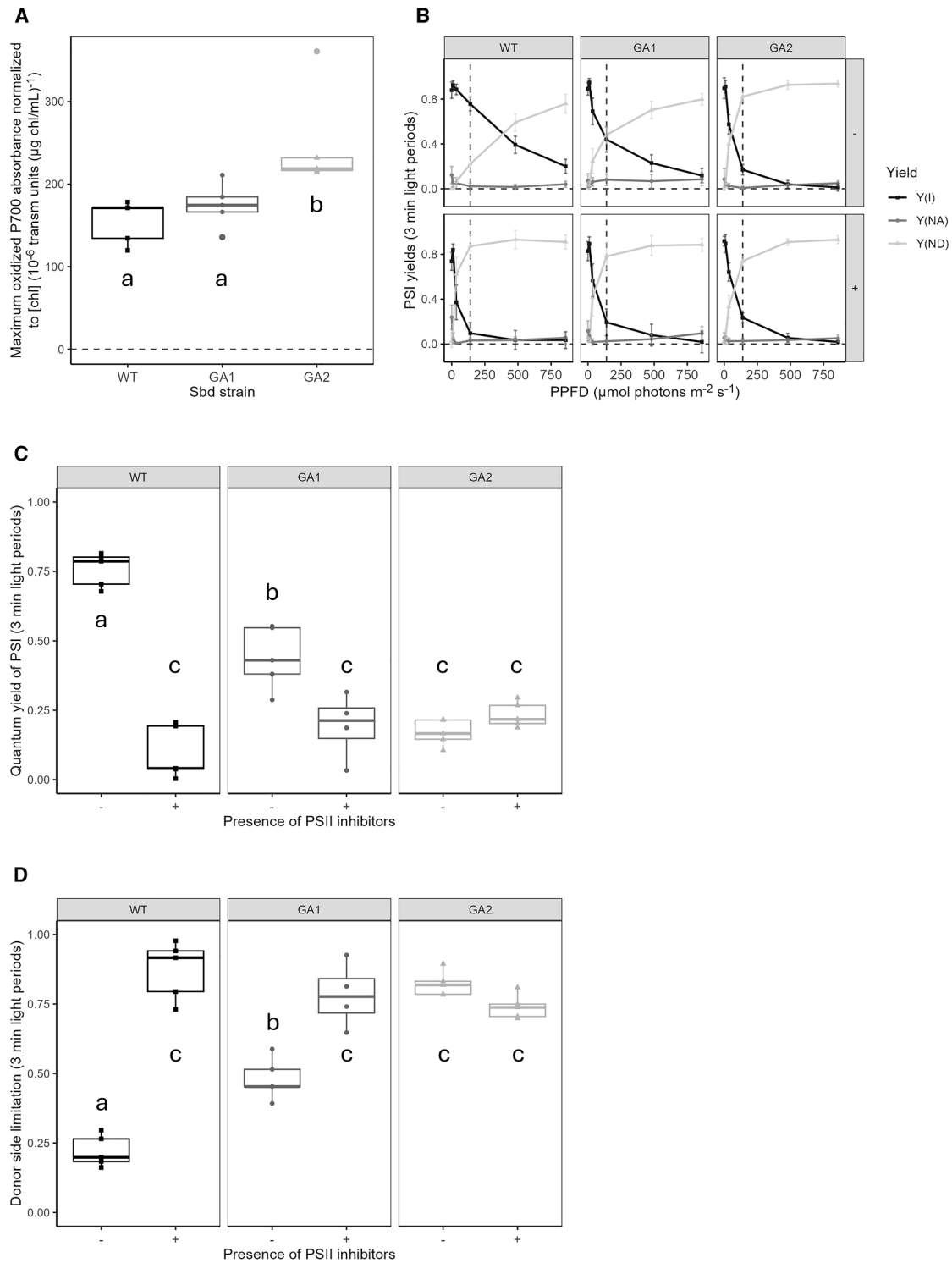


Figure 5. PSI function in the mutants

(A) The maximum P700 absorbance normalized to the concentration of chlorophyll as an indication of the relative amount of PSI.

(B) PSI yields at different photosynthetic photon flux densities. Y(I) indicates quantum yield of photochemical energy conversion, Y(NA) indicates quantum yield of non-photochemical energy dissipation due to acceptor-side limitation, and Y(ND) indicates quantum yield of non-photochemical energy dissipation due to

(legend continued on next page)

Table 1. Characterization of PSII and PSI for mutant and WT strains

Photosystem	Parameter	Wild type	GA1 (PsbI-deficient)	GA2 (PsbE-deficient)
PSII	PSII maximum quantum yield	0.55	0.25	0
	maximum relative electron transport rate through PSII	normal	as wild type – 3 s	0
			< wild type – 3 min	
PSI	active PSI level (per chl)	normal	normal (n.s.d. from wild type)	> wild type
	PSI quantum yield	normal	< wild type	< wild type and < GA1
	+ effect of PSII inhibitors	strong decrease	slight decrease	none
	donor side limitation of PSI	some	> wild type	> wild type and > GA1
	+ effect of PSII inhibitors	strong increase	slight increase	no increase

The table summarizes the results of the different measurements made.

● **EXPERIMENTAL MODEL DETAILS**

- Dinoflagellate starting strain
- Routine culturing
- Growth curves and cell counting methods
- Spot growth

● **METHOD DETAILS**

- DNA analysis
- Spectroscopic methods

● **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cub.2025.10.029>.

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donor-side limitation. The lower half shows the same as the upper half in the presence of acceptor-side (DCMU) and donor-side (hydroxylamine) inhibitors of PSII. “–” indicates the absence of these inhibitors, while “+” indicates their presence.

(C and D) Comparison of the values of Y(I) (C) and Y(ND) (D) at 140 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Different letters (a, b, and c) indicate values that are statistically significantly different (a from b, etc., $p < 0.05$, Dunn’s test for (A), and Tukey’s test for (C and D) (normality checked using the Shapiro-Wilk test), $n = 4$ –5 for all panels). Error bars (in B) represent standard deviation (SD).

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dinoflagellate strain		
<i>Symbiodinium microadriaticum</i> CCMP2467	Lab stock	Obtained from C Voolstra, KAUST, Saudi Arabia
Chemicals, peptides, and recombinant proteins		
Coral Pro Salt	Red Sea Aquatics Ltd	SU_113983/R11230
Phytoplankton Nutrient Guillard's f/2 Medium	Reefphyto UK	N/A
Difco™ Casamino acids, Vitamin Assay	ThermoFisher (Gibco)	228820
Lugol solution	Sigma-Aldrich	32922
Ficoll® 400	Sigma-Aldrich	F4375
(3-(3,4-dichlorophenyl)-(1,1-dimethylurea) (DCMU)	Sigma-Aldrich	D2425
Hydroxylamine (HA)	Sigma-Aldrich	8.14441
Critical commercial assays		
Library preparation and DNA Sequencing	Novogene, Cambridge, UK	N/A
Nucleospin Tissue Mini Kit	Macherey-Nagel, Germany	740952.50
AllTaq Master Mix Kit	QIAGEN, Germany	203144
Deposited data		
Sequence reads from wild-type and minicircle deficient strains	This study	BioProject: PRJNA1330361
Oligonucleotides		
See Table S1	N/A	N/A
Software and algorithms		
Geneious	www.geneious.com	N/A
Biorender (for graphical abstract created by ER)	https://www.BioRender.com/yi9iy0j	N/A

EXPERIMENTAL MODEL DETAILS

Dinoflagellate starting strain

A lab stock was used that was derived from cultures of the dinoflagellate *Symbiodinium microadriaticum* CCMP2467 obtained from Christian Voolstra (KAUST, Saudi Arabia).

Routine culturing

Cells were cultured in f/2 medium, prepared by dissolving 33.6 g l⁻¹ Coral Pro Salt (Red Sea Aquatics Ltd, UK) and 0.5 g l⁻¹ tricine in Milli-Q® Ultrapure Water. The pH of the solution was adjusted to pH 7.6–7.8 with NaOH. This artificial sea water solution was autoclaved prior to the addition of 1.5 ml of a sterile concentrated f/2 nutrient supplement (Reefphyto, UK) per litre of solution. When required, 200 g l⁻¹ glucose was added to a final concentration of 5 g l⁻¹ (0.5% w/v) and casamino acids (amino acids derived from casein hydrolysis, Difco Casamino Acids, Vitamin Assay) to a final concentration of 4 g l⁻¹. The cells were grown without shaking, with illumination (16h light/8h dark) with white light at photosynthetic photon flux density as specified. Culture conditions used specifically for spectroscopic analysis are given below.

Growth curves and cell counting methods

Rapidly growing *S. microadriaticum* WT and GA1 starter cultures were harvested from glucose-containing f/2 medium and grown in f/2 only or f/2+ glucose. *S. microadriaticum* cell densities were measured by haemocytometry, using 10 µl of Lugol's solution (Sigma-Aldrich Company Ltd., UK).

Spot growth

Drops of 10 µl of a *Symbiodinium microadriaticum* WT, GA1, or GA2 cell suspension (5 × 10⁶ cells ml⁻¹) were spotted onto f/2 medium agar plates. The plates were either unsupplemented or supplemented with glucose and casamino acids. To prepare the plates, autoclaved agar (30 g l⁻¹; Select) was mixed with twice-concentrated f/2 medium in filtered artificial seawater. When supplements were

required, glucose and casamino acids were added to the f/2 solution after sterilization by filtration (using 0.22 μm filters) to final concentrations of 5 g l⁻¹ (0.5% w/v) and 4 g l⁻¹ (0.4% w/v), respectively. The cell suspensions were left to dry on the plates under sterile conditions. Cultures were incubated at 26°C with a 16-hour light/8-hour dark cycle at a range of PPFD values.

METHOD DETAILS

DNA analysis

Extraction

DNA from *S. microadriaticum* cultures was isolated using the NucleoSpin® Tissue Mini Kit (Macherey-Nagel, Germany) with modification. 60 mg cells (wet weight) were pelleted by centrifugation of 90 ml stationary-phase culture (3,000 x g, 10 min), resuspended in 1 ml medium, and pelleted again (3,000 x g, 1 min). 200 mg glass beads were added to the 60 mg cells, and the cells were disrupted in a bead mill (2 x 5 min). In addition, the lysis step occurred for 30 minutes.

PCR amplification of minicircles

Primers were designed based on alignments of known *Symbiodinium* minicircle sequences (typically *Breviolum minutum*, *Fugacium kawangutii*, *Cladophora goreauii*, *Cladophora* C3, *Symbiodinium* KB8 and *S. microadriaticum*). Primer sequences are listed in Table S1. The AllTaq® Master Mix Kit (QIAGEN, Germany) was used as per the manufacturer's instructions. PCR products were analysed on a 0.8% agarose gel and stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, US).

Sequencing

All NGS sequencing including library preparation was carried out by Novogene (Cambridge, UK). DNA prepared as described above was used to create libraries and subsequently sequenced to generate 60M raw reads utilizing an Illumina NovaSeq X Plus PE150 workflow. Raw data was filtered to remove adapters and low-quality sequences. Sequences were analysed using Geneious. The filtered raw reads were mapped to coding regions of the various minicircles using the Geneious mapping algorithm. In cases where a minicircle was expected to be present >10,000 mapped sequences were observed, whereas in the mutant examples zero sequences were mapped.

Spectroscopic methods

Culturing

The cells used for spectroscopic measurements were grown at 26°C and at ~5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (16h light/ 8h dark), either in liquid f/2 medium enriched with 5 g l⁻¹ of glucose and 4 g l⁻¹ of casamino acids or on f/2 medium agar plates supplemented in the same way with glucose and casamino acids. In the latter case, cells were resuspended in supplemented liquid f/2 medium before measurements. WT samples were concentrated to 5x10⁶ cells ml⁻¹ and GA1 and GA2 strains were set to a similar chlorophyll concentration. They were all resuspended in f/2 + Ficoll 100 g l⁻¹ for all spectroscopic measurements, except from the measurement of the fluorescence emission spectrum at 77K.

Variable chlorophyll a fluorescence

Variable chlorophyll a fluorescence was assessed using pump and probe fluorimeters: either a SpeedZen imaging system (BeamBio/API, France, for 3 min PSII relative electron transport rate data) or a Joliot-type spectrophotometer (JTS-10, BioLogic, France, for PSII maximum quantum yield and 3 s PSII relative electron transport rate data).⁴⁰ In both cases, blue light (450–470 nm) was used for probe flashes (duration of 280 μs for SpeedZen and 4 μs for JTS measurements), and the resulting fluorescence was measured (with a CCD camera IDS-CP3240-NIR for Speedzen and with a HAMAMATSU S3204-08 Silicon Pin Photodiode for JTS measurements) after filtration through a long-pass red filter (> 685 nm). The actinic light used for saturating pump flashes (150 ms, PPFD >3000 $\mu\text{E m}^{-2} \text{s}^{-1}$) and continuous light periods (either 3 min or 3 s, up to PPFD of 860 $\mu\text{E m}^{-2} \text{s}^{-1}$) was emitted by arrays of LEDs at 660 nm or at 640 nm (for Speedzen or JTS-10 measurements respectively).

The minimal fluorescence value (F_0) was quantified from dark adapted samples, while the maximal fluorescence value (F_m) was recorded after an actinic saturating flash. These values were used to calculate the maximum quantum yield of PSII (F_v/F_m) as follows: $F_v/F_m = (F_m - F_0)/F_m$. The fluorescence at steady state (F_s) was measured on samples exposed to different light periods of increasing intensities, either during 3 s or 3 min. In these conditions, the samples were also subjected to an actinic saturating flash, and probed for the maximum fluorescence from the corresponding actinic light intensity (F_m'). Based on these values, the quantum yield of PSII at that intensity was calculated: $Y(II) = (F_m' - F_s)/F_m'$. The relative electron transport rate through PSII (rETR-PSII) at that intensity was found by multiplying $Y(II)$ by the value of the photosynthetic photon flux density (PPFD) to which the sample was exposed.⁴¹

P700 redox state spectroscopic measurement

The redox state of the reaction centre chlorophyll (P700) can be assessed through its light-induced absorbance changes in the near-infrared.^{41,42} Absorbance measurements were performed with a Joliot-type spectrophotometer (JTS-10), exposing the samples to short (4 μs) detecting flashes at 705 nm. Variation of the redox state of P700 was triggered by exposure to actinic light emitted by LEDs at 660 nm, either through continuous light periods of increasing intensities (3 min, PPFD up to 875 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) or saturating flashes (5 ms, PPFD >3000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). As the electron donor to PSI is cytochrome c_6 instead of plastocyanin in peridinin plastids,⁴³ no further deconvolution was required to assess the redox signal of P700.⁴⁴

Samples subjected to continuous actinic light at different intensities for 3 minutes displayed an absorbance value at steady state (Ps), varying according to the light intensity used. The maximum value of absorbance for samples acclimated to this light intensity

(Pm') was measured by providing a saturating actinic flash. The samples were brought back to the dark for ~450 ms, after which their absorbance value was recorded as Po. The same samples were treated with the PSII inhibitors DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) at 132 μ M (from a stock solution in ethanol) and hydroxylamine (HA) at 2 mM (from a stock solution in H₂O). Subsequently, samples were subjected to the same light sequences, and Po, Ps and Pm' were again measured at different light intensities. For each sample, the strongest value of Pm' (in absence or presence of inhibitors) was selected as the maximum absorbance value (Pm). Based on these values, the quantum yield of PSI at each light intensity was calculated as $Y(I) = (Pm' - Ps)/(Pm - Po)$, the quantum yield of donor side limitation as $Y(ND) = (Ps - Po)/(Pm - Po)$ and the quantum yield of acceptor side limitation as $Y(NA) = (Pm - Pm')/(Pm - Po)$.⁴¹

Electrochromic shift phase A

Electron transport through the photosynthetic complexes is associated with the establishment of an electrochemical gradient ($\Delta\Psi$) between the two sides of the thylakoid membrane. Variation of this gradient induces a shift in the absorbance spectrum of pigments found across the membrane. This process, often referred to as the electrochromic shift (ECS), can be split into 3 phases. Phase A occurs within the first ms after the start of illumination and results from charge separation at the level of photosystems.⁴⁵ As the ECS signal in *Symbiodinium* is linearly related to the intensity of the electric field,⁴⁶ the extent of the phase A of the ECS signal after a saturating flash of light illustrates the amount of the active photosystems in the sample, all inducing one charge separation. A repetition of this measurement after the addition of PSII inhibitors (usually a combination of DCMU and HA) corresponds to the fraction of active PSI in the same conditions, hence allowing us to calculate the proportion of PSI compared to PSII.⁴⁴

ECS phase A signal was determined by assessing the variation of absorbance at 563 nm 500 μ s after a saturating single-turnover flash of 5-6 ns supplied by a Nd:YAG Laser (532 nm, Minilite II, Continuum).⁴⁷ For each sample, the signal of active PSIs and PSIIs was computed from the average of 50 successive flashes interspersed with 1 s breaks. DCMU (132 μ M) and HA (2 mM) were added to the samples and a five-minutes incubation was conducted. The samples were exposed to white light for a few seconds before the measurement of the active PSIs signal through another round of single-turnover flashes. The signal of active PSIIs was calculated from the difference of the signal without inhibitors (PSIs + PSIIs) and the signal with inhibitors (PSIs).

Determination and normalization of chlorophyll concentrations

The cells that had been used for spectroscopic measurements were washed, resuspended in methanol (HPLC grade) and lysed by using a bead beater (MP FastPrep-24 5G, at 4°C, 2x10 s at 6 m/s with a break of 5 min in between). After centrifugation (21130 x g, 5 min, 4°C) to remove cell debris, the absorbance of the samples was recorded at 632, 665 and 750 nm. The value of the absorbance at 750 nm, accounting for remaining debris, was subtracted from the two other values, and the concentration of chlorophylls was calculated.⁴⁸

The maximum value of P700 absorbance variation from the dark (Pm, JTS data) was normalized to chlorophyll concentration, giving an indication of the amount of active PSI in the sample. The value of the signal associated with each active photosystem obtained from the measurement of ECS was also normalized by the chlorophyll concentration of the sample. In order to translate these arbitrary values into more relatable numbers, they were divided by the normalized value associated with the WT on the same day.

QUANTIFICATION AND STATISTICAL ANALYSIS

For the measurements of Fv/Fm (Figure 3), a multiple t-test was used, n=6. For measurements of relative electron transport rates (Figure 3), multiple t-tests or Wilcoxon tests were used, with n=6. For measurements of relative amounts of photosystems (Figure 4), Tukey's test was used, with n=6. Dunn's test was used for measurements of maximum P700 absorbance, and Tukey's test for measurements of quantum yield and donor side limitation, also with n=6 (Figure 5). Significance values are reported in the relevant Figure legends.