

Refactoring the Six-Gene Photosystem II Core in the Chloroplast of the Green Algae *Chlamydomonas reinhardtii*

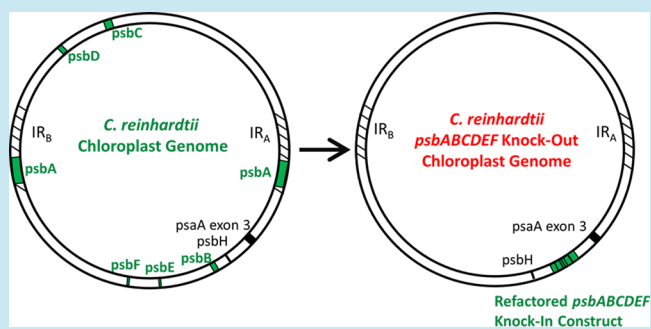
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Supporting Information

ABSTRACT: Oxygenic photosynthesis provides the energy to produce all food and most of the fuel on this planet. Photosystem II (PSII) is an essential and rate-limiting component of this process. Understanding and modifying PSII function could provide an opportunity for optimizing photosynthetic biomass production, particularly under specific environmental conditions. PSII is a complex multisubunit enzyme with strong interdependence among its components. In this work, we have deleted the six core genes of PSII in the eukaryotic alga *Chlamydomonas reinhardtii* and refactored them in a single DNA construct. Complementation of the knockout strain with the core PSII synthetic module from three different green algae resulted in reconstitution of photosynthetic activity to 85, 55, and 53% of that of the wild-type, demonstrating that the PSII core can be exchanged between algae species and retain function. The strains, synthetic cassettes, and refactoring strategy developed for this study demonstrate the potential of synthetic biology approaches for tailoring oxygenic photosynthesis and provide a powerful tool for unraveling PSII structure–function relationships.

KEYWORDS: chloroplast synthetic biology, refactoring, photosystem II, *Chlamydomonas reinhardtii*, photosynthesis engineering, microalgae genetic engineering



The use of synthetic biology to engineer microbes for the production of biofuels and bioproducts is of great interest. Some have proposed using whole engineered synthetic genomes to precisely manipulate host metabolism. Ultimately, this is due to the fact that genes do not act alone but within complex interactive networks and systems, which have evolved together over time. Our greatest deficit of knowledge lies in understanding the extent and complexity of these interactions. One method to relieve this deficit is to break down pathways and complexes into standard DNA sequences of defined structure and function, which can be then easily reassembled in a combinatorial fashion.¹ This will allow the flexibility and modularity of biological pathways and, in particular, large molecular complexes that have been under strong evolutionary constraint to be explored.² By first understanding these building blocks, we can begin to build designed-for-purpose genomes and organisms for the production of specific traits and products.

Recent studies have demonstrated that microalgae are quickly becoming competitive factories for the production of biofuels and bioproducts.^{3–7} Microalgae are highly diverse and are a source of a wide array of bioactive compounds. Microalgae are also fast-growing, genetically tractable, and can be cost-effective and safe hosts for expressing a wide array of recombinant proteins, including human and animal therapeutics, and industrial enzymes.

While the potential of microalgae as a source of a wide range of products is high, optimization of cultivation and processing technologies will be required before algal-derived biofuels and bioproducts can be profitable on a large scale. One of the major limitations to overall growth of algae and plants is the loss of photosynthetic efficiency in response to abiotic stresses. Photosystem II (PSII) is the most susceptible component of the photosynthetic machinery to abiotic stresses. It is directly affected by UV radiation, high salinity, low and high temperature, reactive oxygen species, and high light.^{8,9} Nonetheless, there are examples of photosynthetic organisms that can tolerate each of these conditions independently.^{10,11} The key to abiotic stress tolerance might lie in the PSII genes, as has been shown in several cyanobacteria species.^{12,13} There are an estimated 385 000 photosynthetic species,^{14,15} of which 499 chloroplast and 103 cyanobacteria genome sequences are currently available. By applying synthetic biology approaches, this diversity could be harnessed for engineering desired PSII traits into commercially relevant phototrophs, such as industrial microalgae strains and food crops. Unfortunately, few studies, focused only on individual PSII proteins, have tried to relate the sequence diversity of PSII proteins to specific phenotypes.^{16,17} The aim of this work is to develop the tools that will enable

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synthetic biology of multicomponent modules in *Chlamydomonas reinhardtii* and test whether a complete core PSII complex containing six separate, interdependent proteins can be engineered as a single complex.

PSII is a homodimer supercomplex, with each monomer composed of at least 20 different proteins that interact with up to 100 cofactors.^{18–20} The PSII core complex is defined as the minimal set of proteins required to oxidize water *in vitro*.²¹ The core is composed of D1 (*psbA*) and D2 (*psbD*), which harbor the highly conserved reaction center, flanked by CP47 (*psbB*) and CP43 (*psbC*), which contain the core light-harvesting antenna, and the cytochrome b559 subunits α (*psbE*) and β (*psbF*).^{18,20,22} All of the above proteins are encoded by the plastid genome. The PSII core requires the manganese-stabilizing protein (MSP; PsbO) that caps the water-oxidizing complex (WOC) and is encoded in the nuclear genome. However, PsbO from a wide range of species has been shown to reconstitute PSII from various heterologous hosts *in vitro* and restore the rate of oxygen evolution similar to that of the endogenous protein controls, suggesting a high level of cross-compatibility.^{23,24}

The large number of highly interacting proteins and cofactors, as well as the essential nature of PSII's enzymatic function, has placed a strong evolutionary constraint on PSII, resulting in a frozen metabolic state.² This strong interdependency is seen in the high conservation among the PSII core complex proteins from cyanobacteria to higher plants, even after 2.3–2.5 billion years of evolution.^{25,26} This conservation is higher in the core membrane-spanning proteins compared to that in more soluble or peripheral PSII proteins, such as PsbO or the external light-harvesting complex proteins (LHCII).^{2,25–27}

Altering single PSII proteins by random or scanning mutagenesis would disrupt this frozen metabolic state, likely resulting in an enzyme that lacks one or more of the emergent properties of the whole complex. Furthermore, it would require 20²⁰⁹⁶ amino acid substitutions to scan all possible amino acid combinations from the six-protein core complex (20 amino acid alternatives, 2096 residues constitute the PSII core of *C. reinhardtii*). In contrast, we propose to take advantage of the readily available natural PSII sequence diversity to engineer heterologous core complexes as a whole while maintaining the protein–protein interactions that have evolved over billions of years. Toward this end, we undertook a genome refactoring approach, which consisted of deleting all six core PSII genes and then arranging them into a single synthetic construct for easier manipulation. This strategy has been previously found to be effective for engineering whole genomes and metabolic pathways in viruses and bacteria.^{28–34} In the present article, we generated six *C. reinhardtii* (eukaryotic microalgae) strains lacking one to six PSII core genes (*psbABCDEF*). We then complemented each of the six strains carrying deletions with three independent DNA constructs, each one containing the complete set of PSII core genes from either *C. reinhardtii*, *Volvox carteri*, or *Scenedesmus obliquus* (*Chlorophyceae* green algae). The refactored core complexes from the three algae could restore PSII activity in all of the knockout strains.

RESULTS AND DISCUSSION

Generation of PSII Deletion Strains. As described in work from previous authors, homologous recombination in *C. reinhardtii* chloroplast proved to be very efficient for both deleting genes and recycling the selection markers used for this

purpose.³⁵ Using this strategy, six PSII deficient strains were generated that lacked *psbA*, *psbAD*, *psbADC*, *psbADCB*, *psbADCBE*, and *psbADCBEF* (Figure 1). The six genes are

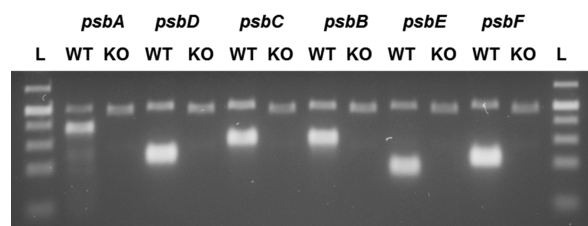


Figure 1. Confirmation of PSII gene deletions in the $\Delta psbADCBEF$ strain. The upper band corresponds to an amplicon from the 16S rRNA gene as a control within the same PCR reaction. The lower bands correspond to amplicons from the corresponding PSII genes. WT: wild-type strain. KO: $\Delta psbADCBEF$ strain. L: Generuler 1 kb Plus DNA Ladder, in which the strongest band corresponds to 500 bp (Thermo Scientific).

present in different loci in the genome (Supporting Information, Figure S2), so each deletion required a specific construct to remove the gene by homologous recombination. The order of deletion was based on the relative conservation of each protein, with D1 and D2 being the most conserved and the cytochrome b559 subunits α (*psbE*) and β (*psbF*) being the least,² and the fact that the AD, BC, and EF proteins each contribute as functional pairs to specific purposes within PSII.^{22,26}

In a previous study, heterologous PSII coding sequences introduced into the *C. reinhardtii* chloroplast genome were eventually replaced by the corresponding endogenous PSII genes due to recombination between identical regulatory regions.³⁶ Our PSII rescue vectors also include the individual endogenous regulatory regions from the PSII genes. Therefore, to minimize the risk of recombination, our primary deletion strategy was to remove the PSII coding sequences and their 5' and 3' regulatory regions. However, the regulatory regions of *psbE* and *psbF* appeared to regulate other photosynthesis-related genes in their original location, as the PSII rescue vectors failed to restore photosynthetic activity in these deletion strains. To overcome this problem, we took an iterative approach to identify the maximum deletable fragment of each of these two genes. The best way to determine if a deletion affects the expression of adjacent genes is by determining if complementation with the deleted fragment at a different site restores photosynthetic growth. Accordingly, we modified our deletion vectors to not include either the 5' or 3' regulatory elements for each of the two PSII genes. This strategy established that the 3' untranslated regions (UTR) of *psbE* and *psbF* were essential for the expression of other photosynthesis-related genes in their original location; hence, those regulatory elements could not be deleted. The *psbF* 3' UTR is likely to affect the expression of *psbL*, which is immediately downstream of *psbF* and is part of the same operon.³⁷ The off-target function of the *psbE* 3' UTR is more difficult to predict since the closest known gene (*rps9*) is located 855 bp downstream of the *psbE* stop codon. Moreover, *rps9* codes for a plastid ribosomal protein, and its loss-of-function is lethal in plants.³⁸ Deletion of the *psbE* 3' UTR in *C. reinhardtii* was not lethal, but it impeded the restoration of photosynthesis.

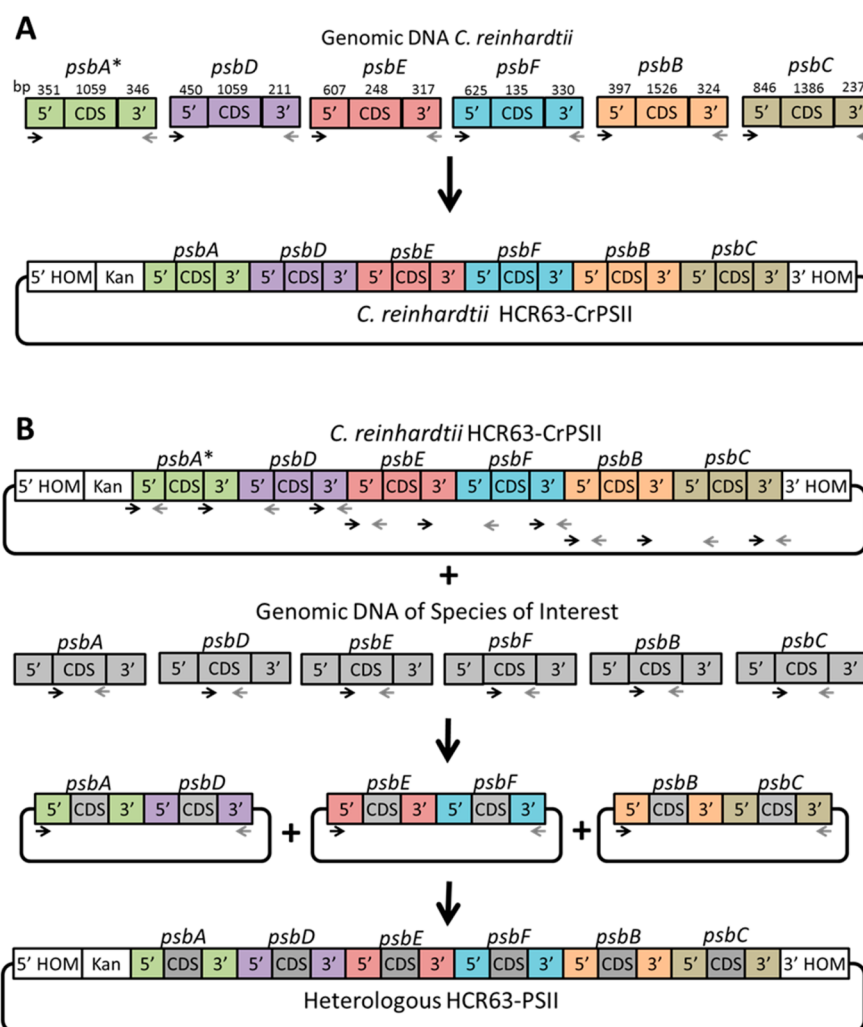


Figure 2. USER assembly of six-gene PSII expression vectors. (A) Assembly of *C. reinhardtii* derived vector HCR63-CrPSII. (B) Assembly of *V. carteri* and *S. obliquus* vectors HCR63-VcPSII and HCR63-SoPSII. Arrows represent USER cloning PCR primers. 5' HOM and 3' HOM: regions for homologous recombination into the chloroplast genome. Kan: kanamycin resistance cassette. **psbA* CDS from *C. reinhardtii* corresponds to an intron-less version.

All gene deletion strains grew well in TAP medium (containing a reduced carbon source), and they were not light-sensitive. Supporting Information Table S1 shows the exact genome positions that were deleted and explains the rationale behind each deletion site.

Construction of Six-Gene PSII Rescue Vectors. We constructed vectors containing the six core PSII genes from three green algae species: *C. reinhardtii*, *V. carteri*, and *S. obliquus*. *C. reinhardtii* PSII served as a control and for assessing the viability of our knockout strains. *V. carteri* was chosen as a closely related specie from the same order as *C. reinhardtii* (*Volvocales*). *S. obliquus* belongs to a different order (*Sphaeropleales*), so it only shares the same class with *C. reinhardtii* (*Chlorophyceae*).³⁹ Supporting Information Table S2 shows the amino acid sequence identities of the PSII proteins, which correlate with the proposed phylogenetic relationships. The close relatedness of these two algae to *C. reinhardtii* is also reflected on the codon usage bias for these genes, measured as the codon adaption index (CAI)⁴⁰ (Supporting Information Table S2). Given the codon usage similarities, PSII coding regions were amplified for cloning using native genomic DNA of *V. carteri* and *S. obliquus*, instead of codon-optimizing and synthesizing the heterologous genes. It is also important to note

that none of the heterologous PSII genes contain introns (except *S. obliquus psbA*, which was chemically synthesized without introns).

All PSII expression constructs utilize the *C. reinhardtii* 5' and 3' regulatory regions for driving the expression of their corresponding coding sequences because previous studies demonstrated that exogenous 5' UTRs are poorly recognized in *C. reinhardtii* chloroplasts.⁴¹ The length of the regulatory regions used in our expression vectors and the references reporting their transcription start sites are shown in Supporting Information Table S3.

All expression vectors were constructed by the USER cloning technique. This technique allowed the assembly of up to 10 PCR products simultaneously, without the need for restriction sites.^{42,43} Additionally, the technique permits the same primers and PCR products to be used when they are shared between different constructs. In this case, the same primers and fragments for the regulatory regions were used for cloning all PSII expression vectors. It is worth noting that Gibson assembly offers comparable advantages and potential for assembling a similar number of fragments in a single reaction.⁴⁴ However, USER fusion was a more cost-efficient technique in our laboratory.

We efficiently assembled the six genes for our *C. reinhardtii* PSII construct in a single six-fragment-USER-fusion step (Figure 2A). Construction of the PSII vectors for the other two algae in one step proved to be inefficient since they required assembly of 13 fragments. Therefore, we generated three subclones, each consisting of two CDS and their corresponding UTRs (five fragments) (Figure 2B). The three pairs of complete genes were then amplified and assembled in a single step into the final vector. This strategy proved to be less time-consuming and more reliable, given the high fidelity of the PfuX7 polymerase used in this study.⁴⁵ The three generated vectors were named HCR63-CrPSII, HCR63-VcPSII, and HCR63-SoPSII and carried the six PSII coding sequences from *C. reinhardtii*, *V. carteri*, and *S. obliquus*, respectively.

Transformation of Deletion Strains with Complementing Gene Sets and Functional Reconstitution of PSII. The three PSII rescue vectors were used to transform the six different PSII deletion strains, resulting in 18 possible combinations. The deletion strains were readily transformed and selected on kanamycin-containing TAP medium plates (with reduced carbon source). Transformants were then tested for autotrophic growth by transferring individual clones to HSM medium (without reduced carbon source). All 18 tested combinations resulted in rescue of photosynthesis (Supporting Information Figure S3).

For the analysis of PSII function, it is important to consider that the synthesis and assembly of active PSII complexes is a tightly regulated process.⁴⁶ Loss of any one of the six PSII core proteins results in absence or severe reduction of accumulation of the other five. The same is true for most of the non-core PSII components.^{47–49}

One of the most widely used and accepted assays for assessing the activity of PSII for light utilization is the ratio between the variable chlorophyll fluorescence and the maximum chlorophyll fluorescence of dark adapted samples, called quantum yield (Q_y , F_v/F_m). This measurement is proportional to the amount of PSII available for active photochemistry.⁵⁰ The Q_y of all generated strains is shown in Table 1. The strains reconstituted with the *C. reinhardtii* PSII

components and tRNA genes associated with these PSII loci (Supporting Information Table S1). Additionally, PSII genes that are transformed into a new site might be misregulated because their expression cassettes could be missing unidentified cis-acting DNA elements. Furthermore, expression of the transformed genes might be affected when they are no longer part of an operon for which post-transcriptional regulation is dependent upon polycistron maturation.⁵¹ Given this complex nature of protein expression in plastids, it is not surprising then that the lowest Q_y was observed in complemented strains $\Delta psbADCBE$ and $\Delta psbADCBEF$, which have accumulated the largest number of exogenous genetic modifications. Nonetheless, it is encouraging that we could recover $85.7 \pm 1.3\%$ PSII functionality in our six-gene refactored *C. reinhardtii* complemented strain (Table 1).

Interestingly, the highest Q_y corresponded to the *C. reinhardtii* reconstituted $\Delta psbADC$ strain and not the strains with fewer genetic modifications: $\Delta psbA$, $\Delta psbAD$, and $\Delta psbADC$. *psbB* is at the beginning of an operon followed by *psbT* and *psbH*, which also code for components of PSII.⁵² The complemented strains $\Delta psbA$, $\Delta psbAD$, and $\Delta psbADC$ might have a lower Q_y because they contain two *psbB* copies (the endogenous and the one from HCR63-CrPSII). Expression of two *psbB* genes could be sequestering an increased amount of the post-transcriptional and translational factors (e.g., Mbb1) that are shared and required for the expression of the other genes from this endogenous operon.⁵² Thus, limited expression of *psbT* and *psbH* genes might be reducing the number of active PSII (Q_y) in the $\Delta psbA$, $\Delta psbAD$, and $\Delta psbADC$ strains.^{53,54}

The Q_y from the strains reconstituted with *V. carteri* and *S. obliquus* PSII core constructs follow a similar trend as that of the strains reconstituted with the endogenous *C. reinhardtii* genes. The first three strains, $\Delta psbA$, $\Delta psbAD$, and $\Delta psbADC$, show a steady but minor decrease in Q_y . The reconstituted $\Delta psbADC$ strains exhibit a steep Q_y decrease. The Q_y values remain stable for both complemented $\Delta psbADCBE$ strains. Finally, there is a less pronounced Q_y decrease when the $\Delta psbADCBEF$ strain is rescued. The heterologous PsbB (CP47) and/or PsbC (CP43) proteins were predicted to be the most limiting components for achieving PSII complementation because they are the largest proteins of the core complex, they are less conserved than the D1/D2 pair² (Supporting Information Table S2), and they directly interact with the less conserved Light-Harvesting II complex surrounding PSII.^{27,55}

Furthermore, a previous study demonstrated that the spinach *psbB* gene was not able to rescue a *Synechocystis* sp. PCC6803 $\Delta psbB$ strain. Chimeric cyanobacteria-spinach CP47 proteins were able to rescue PSII function, but they did so only up to 40% of that of wild-type.⁵⁶ Finally, the apparent Q_y reduction observed when strain $\Delta psbADCBEF$ was complemented is also likely to be related to the fact that PsbF has the lowest amino acid sequence identity out of the six core proteins from *V. carteri* and *S. obliquus* (Supporting Information Table S2).

Overall, our results with heterologous PSII core components support the fact that there are still limiting interactions with other constituents of PSII, which is composed of at least 14 proteins in addition to the core subunits.¹⁹ PsbO is one candidate for generating these limiting interactions because it is less conserved than the six core proteins² and interacts directly with D1, D2, CP43, CP47, and PsbE.^{23,24} Studies are ongoing to determine if expression of heterologous PsbO proteins could further increase the Q_y of strains carrying the corresponding heterologous PSII core centers.

Table 1. Photosystem II Quantum Yields (Q_y) of Transformed Strains According to Variable Fluorescence Measurements (F_v/F_m)^a

Strain	Construct		
	<i>C. reinhardtii</i> HCR63-CrPSII	<i>V. carteri</i> HCR63-VcPSII	<i>S. obliquus</i> HCR63-SoPSII
<i>ApsbA</i>	0.72 ± 0.01	0.72 ± 0.02	0.70 ± 0.01
<i>ApsbAD</i>	0.71 ± 0.02	0.69 ± 0.01	0.69 ± 0.01
<i>ApsbADC</i>	0.71 ± 0.00	0.66 ± 0.01	0.66 ± 0.01
<i>ApsbADC</i> B	0.74 ± 0.01	0.51 ± 0.01	0.46 ± 0.00
<i>ApsbADC</i> BE	0.66 ± 0.01	0.48 ± 0.02	0.46 ± 0.01
<i>ApsbADC</i> BEF	0.66 ± 0.01	0.41 ± 0.01	0.43 ± 0.01
Wild-type	0.77 ± 0.01		

^aA higher Q_y is indicative of a higher proportion of active PSII complexes.

gene sets (first row) showed the overall highest Q_y , as expected. Nonetheless, all of these strains had a somewhat lower Q_y than the untransformed WT control. Q_y decreased as more genetic modifications were added to the strains. This might be due to off-target effects when endogenous loci are disrupted by the deletion of the PSII targeted genes, as some of these deletions have been predicted to affect operon

The Light-Harvesting Complex II (LHCII) surrounding PSII is also a candidate for incompatibility since it shows much greater variability across photosynthetic organisms than that of the PSII core. LHCII from green chloroplasts is composed of several proteins, each encoded by multiple nuclear gene families in a single organism. Additionally, there is no crystal structure of LHCII bound to PSII that could allow for precise identification of interactions between LHCII and the inner light-harvesting antennas, CP43 and CP47.²⁷ The solution for this potential problem would be to engineer heterologous PSII along with its corresponding LHCII. This work is, however, beyond the scope of the present study.

Accumulation of Core Proteins of Reconstituted PSII. ELISA assays were performed in order to quantitate the accumulation of four of the PSII core proteins (D1, D2, CP43, and CP47) in all 18 reconstituted strains (Table 2). Because

Table 2. ELISA Results for Quantification of D1, D2, CP43, and CP47 Proteins from All Strains Generated in This Study^a

Construct	Strain	Protein			
		D1	D2	CP43	CP47
<i>C. reinhardtii</i>	<i>ApsbA</i>	104.7 ± 7.5	104.3 ± 5.8	101.4 ± 2.6	97.1 ± 5.6
HCR63-CrPSII	<i>ApsbAD</i>	88.6 ± 10.2	96.1 ± 6.6	92.5 ± 5.0	99.6 ± 3.3
	<i>ApsbADC</i>	57.3 ± 2.6	76.9 ± 3.9	84.1 ± 4.6	78.4 ± 3.8
	<i>ApsbADCB</i>	77.4 ± 6.4	85.8 ± 5.1	89.7 ± 2.9	91.2 ± 3.4
	<i>ApsbADCBE</i>	52.6 ± 4.0	71.7 ± 3.3	78.2 ± 1.4	82.4 ± 1.5
	<i>ApsbADCBEF</i>	57.4 ± 3.1	63.3 ± 1.8	67.0 ± 1.8	73.7 ± 3.1
<i>V. carteri</i>	<i>ApsbA</i>	45.5 ± 0.6	40.7 ± 1.0	54.4 ± 1.8	44.7 ± 1.3
HCR63-VcPSII	<i>ApsbAD</i>	46.4 ± 0.8	43.9 ± 1.5	57.3 ± 2.9	46.7 ± 1.4
	<i>ApsbADC</i>	27.0 ± 1.3	25.5 ± 1.4	30.5 ± 0.5	28.1 ± 1.3
	<i>ApsbADCB</i>	28.9 ± 2.7	27.7 ± 1.1	22.9 ± 0.7	24.0 ± 1.2
	<i>ApsbADCBE</i>	19.6 ± 0.5	19.1 ± 0.4	18.5 ± 0.7	13.0 ± 0.4
	<i>ApsbADCBEF</i>	16.9 ± 1.8	18.9 ± 0.5	21.8 ± 1.1	13.6 ± 0.6
<i>S. obliquus</i>	<i>ApsbA</i>	75.7 ± 11.7	66.7 ± 1.8	71.4 ± 4.8	66.9 ± 3.9
HCR63-SoPSII	<i>ApsbAD</i>	57.6 ± 1.8	44.0 ± 1.5	51.0 ± 1.5	46.4 ± 2.1
	<i>ApsbADC</i>	36.4 ± 2.8	31.7 ± 0.9	19.3 ± 0.8	35.6 ± 0.7
	<i>ApsbADCB</i>	25.2 ± 1.8	13.8 ± 0.3	12.1 ± 0.4	11.1 ± 0.4
	<i>ApsbADCBE</i>	21.4 ± 0.1	11.9 ± 0.2	11.0 ± 0.2	9.1 ± 0.3
	<i>ApsbADCBEF</i>	23.1 ± 2.1	10.7 ± 0.4	10.0 ± 0.3	8.2 ± 0.2
	Wild-type	100.0 ± 5.8	100.0 ± 2.0	100.0 ± 3.3	100.0 ± 6.5

^aValues correspond to percentage of wild-type *C. reinhardtii* protein accumulation. ± represents the standard deviation from triplicate wells in the ELISA plate.

there may be differences in antibody affinity to PSII proteins from different species, any interspecies comparisons of ELISA data should be considered qualitative and not quantitative. ELISA results from Table 2 can be quantitatively compared only between strains expressing the same protein from a single species. However, due to the high amino acid identity between species (≥94%; Supporting Information Table S2), any potential effect of antibody specificity is predicted to be minor.

PSII protein accumulation in the *C. reinhardtii* PSII complemented strains showed a decreasing trend as the number of complemented genes increases, correlating with the Qy values of these strains. The only case that seems to favor PSII protein accumulation is the deletion of the endogenous *psbB* gene (HCR63-CrPSII strain Δ *psbADCB*). As discussed above, deletion of the endogenous *psbB* gene might allow for increased expression of other PSII components present in the

endogenous *psbB-psbT-psbH* operon.⁵² Interestingly, for some of the *C. reinhardtii* complemented strains, the D1 protein accumulation levels seem to be lower than those of the other three proteins. We attribute this to the high turnover rate of D1, which is constantly degraded and replaced without the disassembly of the rest of PSII. This process of constant repair is highly regulated at the post-transcriptional and translational levels,^{12,49} and some of those mechanisms could have been affected by the extensive genetic modifications in these strains.

Protein accumulation in the strains complemented with *V. carteri* and *S. obliquus* PSII again correlated with their respective Qy values. As more endogenous genes were replaced by heterologous homologues, the amount of PSII proteins decreased. Unlike in the *C. reinhardtii* rescue strains, the CP43 (PsbC) protein accumulation of *V. carteri* and *S. obliquus* PSII complemented strains tended to vary independently of the others. It has been shown that CP43 can accumulate to some extent without the presence of the other core PSII component proteins. Additionally, CP43 is assembled at the final steps during PSII biogenesis, so the remaining subunits can also accumulate to low levels in its absence.^{46–48} It also appears that the *S. obliquus* complemented strains tend to accumulate more D1 protein relative to the other three subunits. This might be due to its lower amino acid identity, which might make it a less suitable target for D1-specific proteases.^{12,46} Alternatively, the D1 protein itself has been shown to autoattenuate its translation through an interaction with the 5' UTR of *psbA* mRNA.⁴⁹ Perhaps the *S. obliquus* D1 protein is less competent in autoattenuating translation initiation from the *C. reinhardtii psbA* 5' UTR.

Finally, we have demonstrated that the PSII core complex can be treated as a synthetic biology module. As might be expected, heterologous components do not perform and/or accumulate at the same level as the endogenous homologues present in the chassis. In order to partially overcome this, future work will have to consider genomic context of genes of interest beyond the current annotated regulatory regions. Notwithstanding, natural PSII protein variants or synthetic isoforms can now be tested as a complete functional unit using a single and tractable expression vector for complementation of our deletion strains. This system could be readily applied for reverse genetic studies to understand the structure–function relationships within PSII, as we have previously shown with a *C. reinhardtii psbA* deletion strain complemented with *psbA* variants from cyanobacteria.⁵⁷ Additional PSII components could be added to our proposed platform, and/or expression from an artificial plastid chromosome could be implemented,³⁶ in order to improve the quantum yield of chimeric PSIIs. An optimized system could be used to identify PSII variants with improved growth under diverse environmental conditions. These discoveries might be further translated into plant crops in the future.

■ METHODS

Strains, Culture Conditions, Biolistic Transformation, and PCR Screens. *C. reinhardtii* Dangeart 137c mt+ (Chlamydomonas Resource Center, University of Minnesota) was used as the wild-type strain in this study. A 137c mt+ that had its *psbA* genes replaced by a spectinomycin cassette (Rasala, unpublished) was used for the generation of the PSII deletion strains. All strains were grown on TAP medium (containing acetate as a carbon source) at 25 °C in agitated flasks or 1.5% agar plates, except for the PSII quantum yield

experiments. Constant illumination of $65 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ was provided by white fluorescent tubes. Biolistic transformation was performed as described in ref 58. TAP agar plates containing kanamycin ($100 \mu\text{g}/\text{mL}$) were used for selection. PCR screens for confirming gene deletion and genome homoplasmy were executed as in ref 59.

Generation of PSII Deletion Vectors and Strains. For all cloning experiments, the USER Fusion PCR-based ligase-independent cloning technique was used.⁴² PCRs were carried out using in-house-purified PfuX7 polymerase.⁴⁵ Uracil-containing primers were purchased from IDT (Coralville, IA, USA). All PCR fragments were gel-purified using the QIAquick gel extraction kit (Valencia, CA, USA). USER enzyme was purchased from New England Biolabs (Ipswich, MA, USA).

Six vectors were constructed for deleting the six PSII core genes by homologous recombination. All vectors were derived from plasmid HCR2, which contains two USER cloning sites flanking a kanamycin resistance cassette (Supporting Information Figure S1B and Table S4). Each construct consisted of 5' and 3' homology regions (1000 bp each) that were used for targeted recombination and deletion of the PSII loci. The homology regions flanked a kanamycin cassette and a downstream direct repeat sequence (Supporting Information Figure S1C). The direct repeat was identical to one of the homology regions, allowing the kanamycin cassette to be looped out by homologous recombination.³⁵ Primers for assembling the six deletion constructs are shown in Supporting Information Table S5.

The constructs were transformed one at a time, and the transformants were passaged several times on kanamycin plates. PCR was used to confirm that the corresponding PSII gene was deleted from all of the chloroplast genome copies (homoplasmy) (Figure 1). Once the deletion was complete, the selected strain was restreaked in TAP medium without antibiotic to allow recombination between the direct repeat and the appropriate homology region to loop the kanamycin cassette out of the genome.³⁵ The kanamycin sensitive strain was then ready for another round of transformation and deletion. The genes were deleted in the following order: *psbA*, *D*, *C*, *B*, *E*, and *F*. It is worth noting that we tested cotransformation of two deletion constructs, each carrying either kanamycin or spectinomycin selection cassettes, for disrupting two genes simultaneously. However, we could never obtain transformants on double selection plates.

Construction of PSII Expression Vectors. For subcloning purposes, we generated plasmid HCR1, a modified version of pBlueScript II (Agilent, Santa Clara, CA, USA) containing a single USER cloning site within the NotI restriction site (Supporting Information Figure S1A and Table S4).⁶⁰ HCR1 was then used to construct the PSII recipient vector (HCR63), which consisted of two homologous recombination regions (from *C. reinhardtii* plastid genome) flanking a USER cloning site and a kanamycin resistance cassette (Supporting Information Figure S1D). HCR63 allowed us to USER fuse several PSII genes at the same time and subsequently use the resulting vector to transform the chloroplast genome at the neutral site between *psbH* and *psaA* exon 3 (Supporting Information Figure S2) while selecting for kanamycin resistance. We first cloned *C. reinhardtii* endogenous PSII genes into HCR63, generating HCR63-CrPSII. The complete genes (including regulatory elements) were amplified from genomic DNA by PCR and cloned in a single step (Figure 2A). *C. reinhardtii psbA* contains introns, but, in this case, the gene

was amplified from a plasmid containing an intron-less version including its corresponding regulatory regions.⁶¹ PSII expression vectors for *V. carteri* and *S. obliquus* were not produced in a single cloning step. Three subcloning vectors (based on HCR1) were generated for each species, each one containing two heterologous PSII coding sequences with their corresponding *C. reinhardtii* 5' and 3' regulatory regions (Figure 2B). The regulatory regions were amplified from HCR63-CrPSII, and the coding sequences were amplified from their corresponding genomes. None of the CDS contained introns, except *S. obliquus psbA*, which was chemically synthesized without introns (IDT). The three pairs of genes were then amplified from plasmids and USER cloned into HCR63, generating HCR63-VcPSII and HCR63-SoPSII. Primers for assembling the three complementation constructs are shown in Supporting Information Table S6. All PSII genes were fully sequenced before transformation experiments. All transformed *C. reinhardtii* strains were confirmed by PCR amplifying a region unique to the vectors, between the kanamycin cassette and the *psbA* promoter (Supporting Information Figure S2B).

PSII Quantum Yield (F_v/F_m) Measurements. Cells were inoculated into 16 mm test tubes with 6 mL of HSM (minimal medium without a carbon source) up to a concentration of 1×10^5 cells/mL in three replicates per strain. Tubes were placed in a roller drum within a 25 °C growth chamber supplemented with carbon dioxide, under $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of continuous illumination for 1 day. An aliquot of cells was taken from each tube and used for PSII quantum yield measurement using an Aquapen-C PAM fluorometer (Photon System Instruments, Drasov, Czech Republic). Cells were diluted to $4 \mu\text{g}/\text{mL}$ total chlorophyll in HSM supplemented with 5 mM sodium bicarbonate. Two milliliters of cell suspensions were placed in cuvettes for dark adaptation for 5 min before measuring. A 455 nm probe pulse at $0.0243 \mu\text{mol}\cdot\text{m}^{-2}$ per pulse and a saturating pulse at $1500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ intensity gave the highest quantum yield values at this chlorophyll concentration and were used in this study.

Protein Preparation and ELISA. ELISA assays were performed essentially as in ref 62. Algae were streaked in TAP plates and grown for 2 days under $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. Samples were collected and resuspended in PBS-T buffer containing complete protease inhibitor cocktail (Roche, Basel, Switzerland). Disruption was performed by sonication. Total protein was quantified using the Dc protein assay (Bio-Rad, Hercules, CA, USA). 96-well Nunc Maxisorp clear plates (Thermo Scientific, Waltham, MA, USA) were used for all assays except for the D1 protein. Acrowell BioTrace NT plates (Pall, Port Washington, NY, USA) were used for the D1 ELISA assays because they showed better signal-to-noise ratio for this protein. Total protein ($0.5 \mu\text{g}$) diluted in $100 \mu\text{L}$ of 100 mM carbonate/bicarbonate coating buffer, pH 9.6, supplemented with 25% isopropanol⁶³ was loaded per well from each sample in triplicates. Protein binding was done overnight at 4 °C. Blocking was performed for 2 h at room temperature with 10 mg/mL BSA in PBS. PBS without detergent was used for all washing steps. All primary antibodies used were rabbit polyclonal diluted in PBS + 1 mg/mL BSA and incubated for 2 h at room temperature. Anti-D1(C-terminus) and anti-D2 were used at a 1:10000 dilution (Agriser, Vännäs, Sweden). Anti-CP43 and anti-CP47 were generated in our laboratory and used at a 1:20000 dilution. Horseradish peroxidase-conjugated goat anti-rabbit (Sigma, St. Louis, MO, USA) was used as a

secondary antibody and incubated for 2 h at room temperature in a 1:20000 dilution in PBS + 1 mg/mL BSA. The assay was developed using the 1-Step Ultra TMB-ELISA substrate according to the manufacturer's instructions (Thermo Scientific).

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00076.

Additional information about the coding and regulatory sequences used in this study; USER cassette sequences; additional maps of the vectors constructed for this study; all of the primers used for assembling the PSII knockout and complementation vectors (PDF).

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Notes

The authors declare no competing financial interest.

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