Supplementary Information

Epimerization of chlorophyll *a* analogs: effects of the vinyl groups in the A-ring and B-ring of the chlorin macrocycle

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Experimental details on CRISPR/Cas9 genome editing

Genome editing experiments were performed as described in previous reports [S1,S2], with slight modifications. Two 20-nucleotide CRISPR RNA (crRNA) guide sequences (AAAGATTGGCTTCAGCGAAGCGG and TTCAACACGCATCAGTGCAAAGG) were designed using the CHOPCHOP (version 3) webtool [S3]. The crRNAs were custom-synthesized by Integrated DNA Technologies (Coralville, IA, USA). Guide RNAs were prepared by hybridizing 60 pmol of each crRNA with 60 pmol of tracrRNA (Alt-R CRISPR-Cas9 tracrRNA, Integrated DNA Technologies), followed by incubation with 8 μg of Alt-R S. p. HiFi Cas9 Nuclease V3 (Integrated DNA Technologies) to assemble a pair of ribonucleoprotein (RNP) complexes. The expected cleavage sites in the Eg*DVR* coding region of the genomic DNA of *Euglena gracilis* are shown in Figure S1.

To generate knockout strains (ΔEg*DVR*), the two RNPs targeting distinct loci in the first and second exons, respectively (Figure S1), were introduced simultaneously into *E. gracilis* cells using the electroporation procedure described in the next section. This approach resulted in a deletion of approximately 184 bp at the target region, which was expected to disrupt the function of the Eg*DVR* gene (Figure S1).

For electroporation, *E. gracilis* cells cultured in Cramer–Meyer medium (CM medium) [S4] for two weeks were used. A total of 5×10^4 cells were resuspended in 50 μL of fresh CM medium containing 100 μM of each RNP and 200 μM of the ssODN, and electroporated using a GENE PULSER II system at 0.45 kV and 50 μF in a 0.2-cm gap cuvette. Immediately afterward, the cells were transferred to fresh CM medium and incubated at 26 °C in the dark for 12 h, followed by 72 h in a 14L:10D light cycle at 26 °C (primary culture). Clonal strains were obtained from the primary culture by aseptically isolating individual *E. gracilis* cells into culture plates containing Koren–Hutner medium [S5] using a microcapillary method with glass tubes. The targeted genomic region of each clone was PCR-amplified and sequenced to identify successfully edited clones.

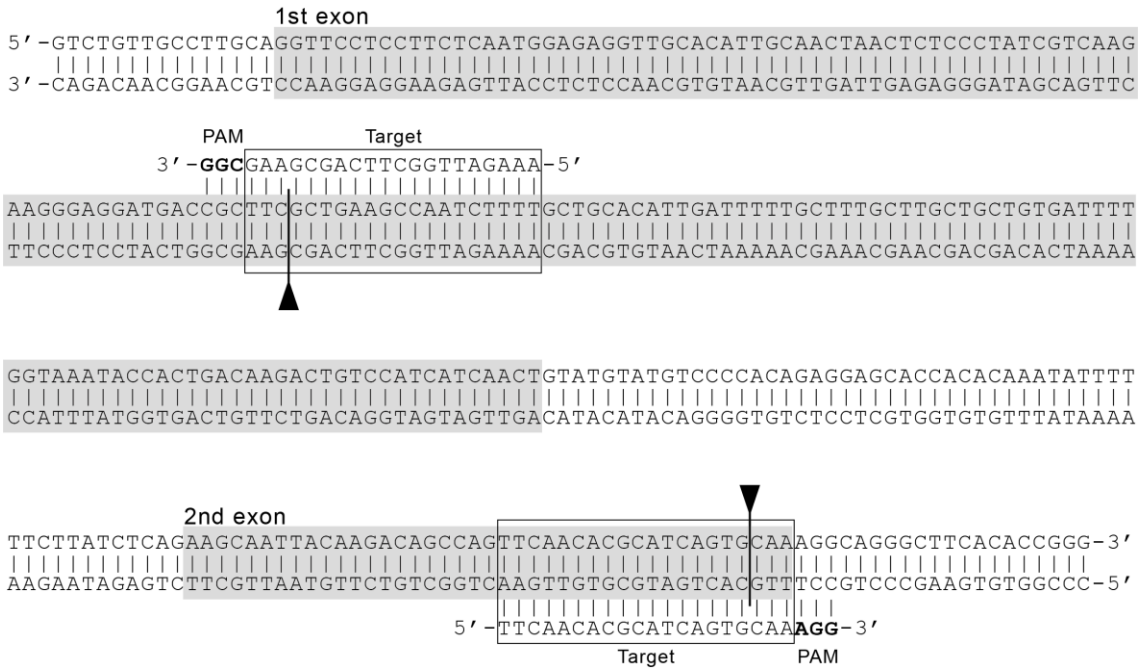
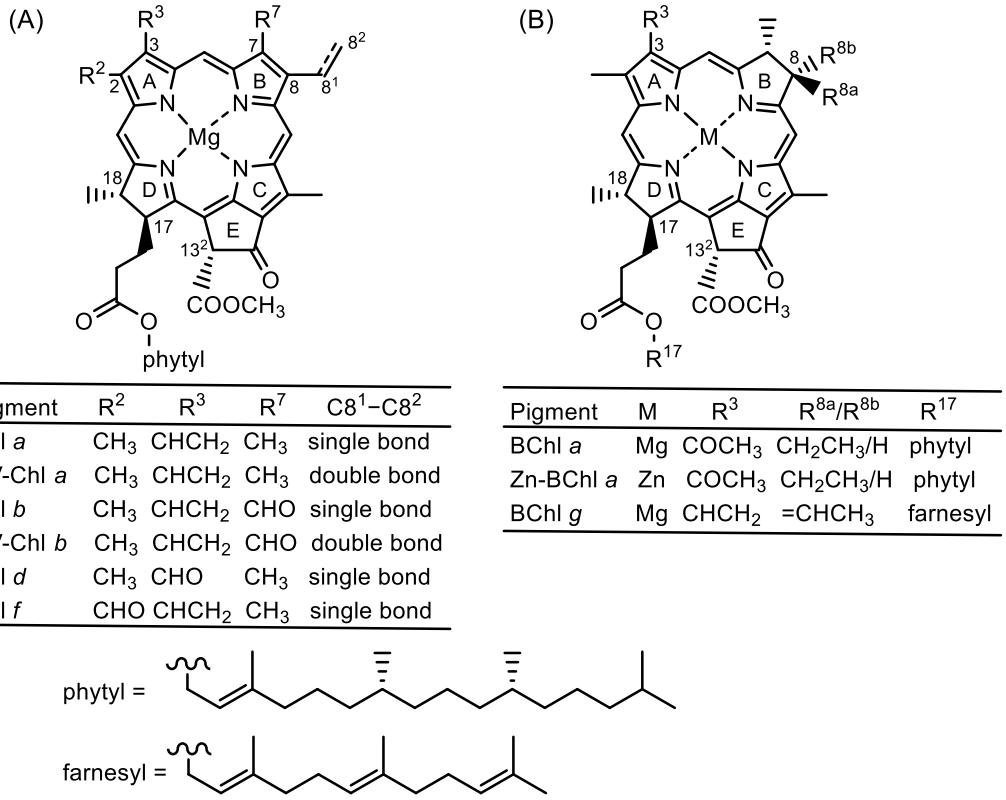


Figure S1. Genomic DNA sequence encompassing the first and second exons of the Eg*DVR* gene, the crRNA sequences used for the CRISPR/Cas9 target sites, and the expected cleavage sites indicated by arrowheads.



Pigment	R^2	R^3	R^7	$C8^1$–$C8^2$
Chl *a*	CH_3	$CHCH_2$	CH_3	single bond
DV-Chl *a*	CH_3	$CHCH_2$	CH_3	double bond
Chl *b*	CH_3	$CHCH_2$	CHO	single bond
DV-Chl *b*	CH_3	$CHCH_2$	CHO	double bond
Chl *d*	CH_3	CHO	CH_3	single bond
Chl *f*	CHO	$CHCH_2$	CH_3	single bond

Pigment	M	R^3	R^{8a}/R^{8b}	R^{17}
BChl *a*	Mg	$COCH_3$	CH_2CH_3/H	phytyl
Zn-BChl *a*	Zn	$COCH_3$	CH_2CH_3/H	phytyl
BChl *g*	Mg	$CHCH_2$	$=CHCH_3$	farnesyl

Figure S2. Molecular structures of naturally occurring Chl pigments possessing a chlorin macrocycle in oxygenic photosynthetic organism (A) and BChl pigments possessing a bacteriochlorin macrocycle in photosynthetic bacteria (B).

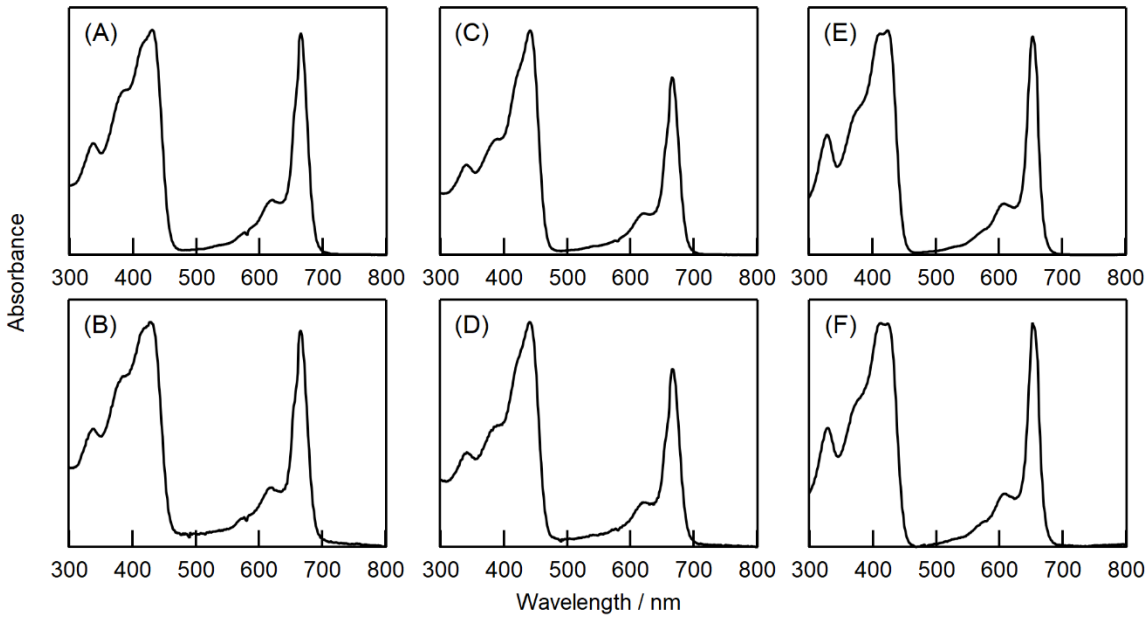


Figure S3. Online electronic absorption spectra in methanol of pigments eluted former (A/C/E) and latter (B/D/F) in HPLC chromatograms of reaction products after incubation of Chl *a* (A/B), DV-Chl *a* (C/D), and mesoChl *a* (E/F) with triethylamine (0.3 M) in diethyl ether at 20°C for 3 h.

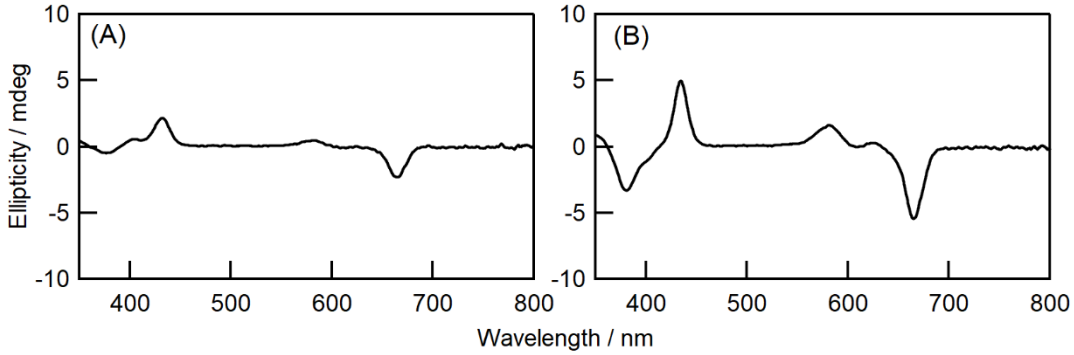


Figure S4. CD spectra in acetone of pigments eluted former (A) and latter (B) in HPLC chromatograms of reaction products after incubation of Chl *a*. The Q_y absorbance of the samples was adjusted to be 0.5.

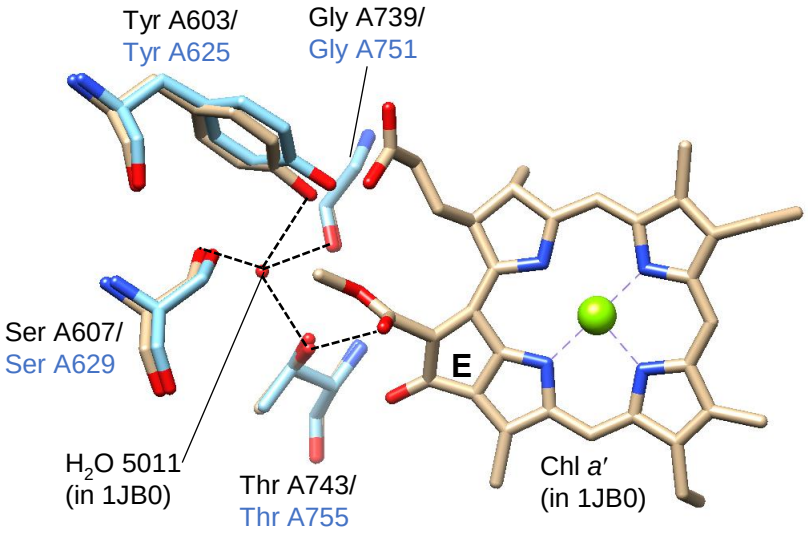


Figure S5. Comparison of the arrangement of amino acid residues participating in the hydrogen-bonding network in the vicinity of the E-ring of Chl *a*′ in PS-I RCs from a cyanobacterium *Thermosynechococcus elongatus* (PDB entry: 1JB0) and *Prochlorococcus marinus* (AlphaFold Protein Structure Database: AF-A2BTA0-F1-v6). Amino acid residues in PS-I RCs of *Thermosynechococcus elongatus* and *Prochlorococcus marinus* are colored gold and sky blue, respectively. Hydrogen-bonds in PS-I RC of *Thermosynechococcus elongatus* are shown by broken lines.

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Prochlorococcus     SLGFRFPCDGPGRGGTCQVSSWDHVFLALFWMYNCLSIVIFHFSWKMQSDVWGL------   646
cyanobacterium      NLGFRFPCDGPGRGGTCQVSGWDHVFLGLFWMYNCISVVIFHFSWKMQSDVWGTVAPDGT   630
plant               NLGFRFPCDGPGRGGTCQVSAWDHVFLGLFWMYNSISVVIFHFSWKMQSDVWGTINDQGV   633
green algae         NLGFRFPCDGPGRGGTCQVSAWDHVFLGLFWMYNSLSIVIFHFSWKMQSDVWGTVTASGV   627
red algae           NLGFRFPCDGPGRGGTCQVSSWDHVFLGLFWMYNCISVVIFHFSWKMQSDVWGTVSQNSL   627

Prochlorococcus     ----TGGNFAQSSITINGWLRDFLWAQASQVLTSYGQSISMYGLMFLGAHFIWAFSLMFL   702
cyanobacterium      VSHITGGNFAQSAITINGWLRDFLWAQASQVIGSYGSALSAYGLLFLGAHFIWAFSLMFL   690
plant               VTHITAGNFAQSSITINGWLRDFLWAQASQVIQSYGSSLSAYGLFFLGAHFVWAFSLMFL   693
green algae         -SHITGGNFAQSANTINGWLRDFLWAQSSQVIQSYGSALSAYGLIFLGAHFVWAFSLMFL   686
red algae           VSHVVGGNFSQSAITINGWLRDFLWAQASQVIQSYGSSISAYGLMFLAAHFIWAFSLMFL   687

Prochlorococcus     FSGRGYWQELFESIVWAHNKLKVAPTIQPRALSITQGRAVGVTHFLVGGIATTWAFFHAR   762
cyanobacterium      FSGRGYWQELIESIVWAHNKLKVAPAIQPRALSIIQGRAVGVAHYLLGGIATTWAFFLAR   750
plant               FSGRGYWQELIESIVWAHNKLKVAPATQPRALSIVQGRAVGVTHYLLGGIATTWAFFLAR   753
green algae         FSGRGYWQELIESIVWAHNKLKVAPAIQPRALSITQGRAVGVAHYLLGGIATTWSFFLAR   746
red algae           FSGRGYWQELIESIVWAHSKLKIAPTIQPRALSITQGRAVGAAHYLLGGIATTWAFFLSR   747

Prochlorococcus     LFGLG   767
cyanobacterium      IISVG   755
plant               IIAVG   758
green algae         IISVG   751
red algae           AISIG   752
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Figure S6. Sequence alignment of the C-terminal region of PsaA polypeptides of *Prochlorococcus* (UniProt ID: A2BTA0), cyanobacterium (UniProt ID: P0A405), plant (UniProt ID: P05310), green algae (UniProt ID: P12154), and red algae (UniProt ID: Q9TLQ5). Amino acid residues participating in the hydrogen-bonding network in the vicinity of the E-ring in Chl *a*′ are colored red.

REFERENCES

S1. Nomura, T., Yoshikawa, M., Suzuki, K. & Mochida, K. (2020). Highly efficient CRISPR-associated protein 9 ribonucleoprotein-based genome editing in *Euglena gracilis*. *STAR Protoc.*, *1*, 100023.

S2. Nagamine, S., Oishi, R., Ueda, M., Sakamoto, T. & Nakazawa, M. (2024). Genome editing-based mutagenesis stably modifies composition of wax esters synthesized by *Euglena gracilis* under anaerobic conditions. *Bioresour. Technol.*, *410*, 131255.

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S5. Koren, L. E.& Hutner, S. H. (1967). High-yield media for photosynthesizing *Euglena gracilis* z. *J. Protozool.*, *14*, 17.