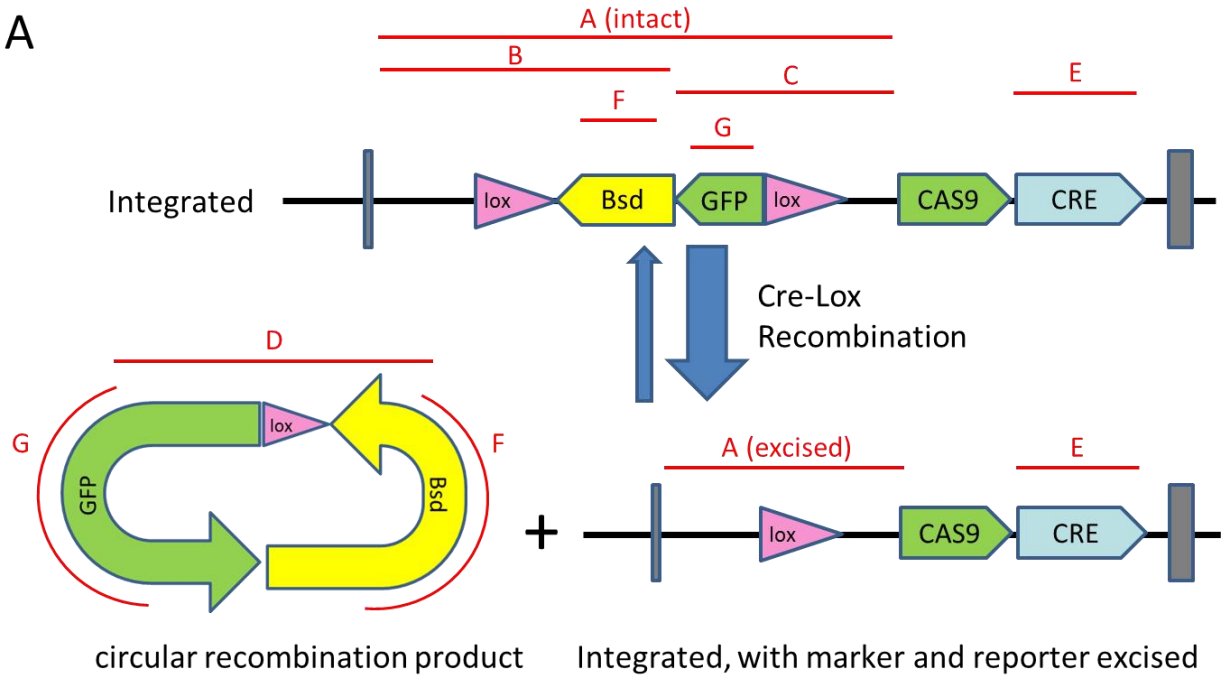
SI appendix:

Supplementary figures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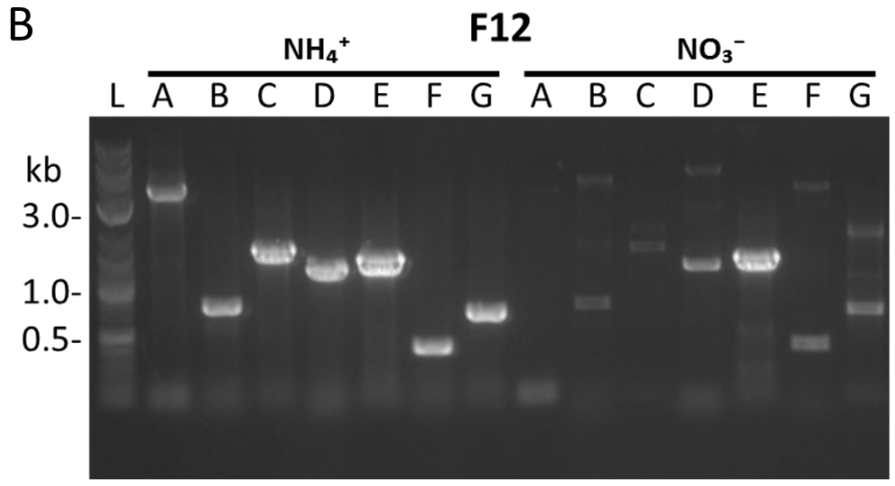
A (intact)
B
C
E
F
G
Integrated
lox
Bsd
GFP
lox
CAS9
CRE
Cre-Lox
Recombination
D
G
F
GFP
lox
Bsd
A (excised)
E
lox
CAS9
CRE
circular recombination product
Integrated, with marker and reporter excised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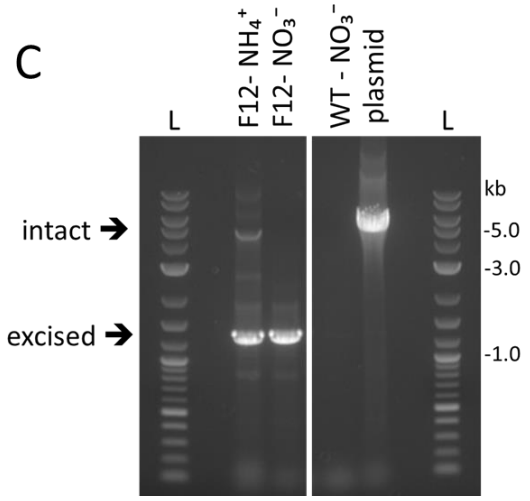
F12
NH4+
NO3−
L A B C D E F G A B C D E F G
kb
3.0-
1.0-
0.5-


C
L
F12- NH4+
F12- NO3−
WT - NO3−
plasmid
L
kb
-5.0
-3.0
-1.0
intact
excised

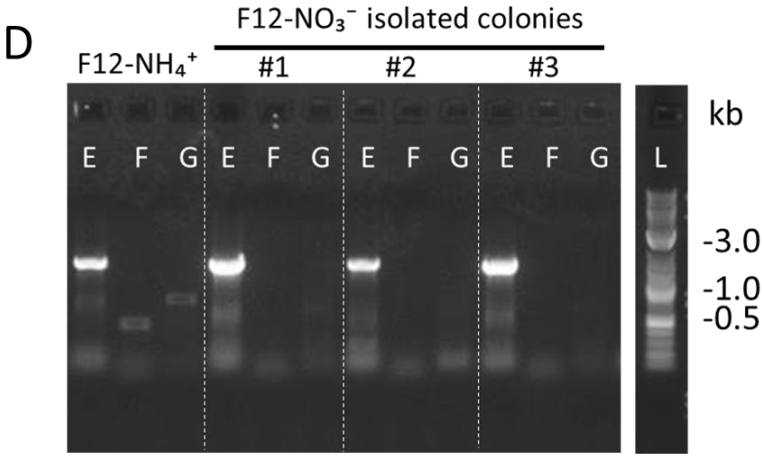
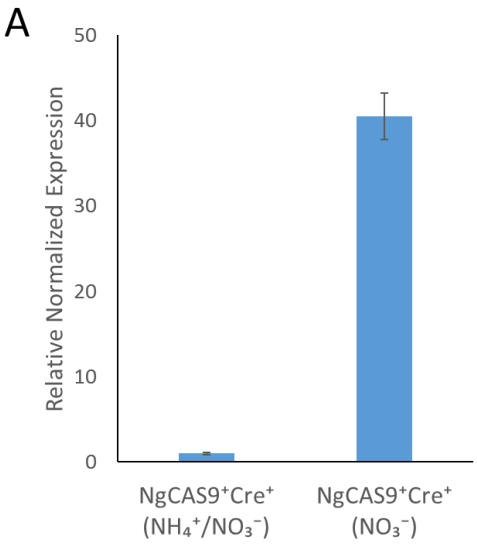
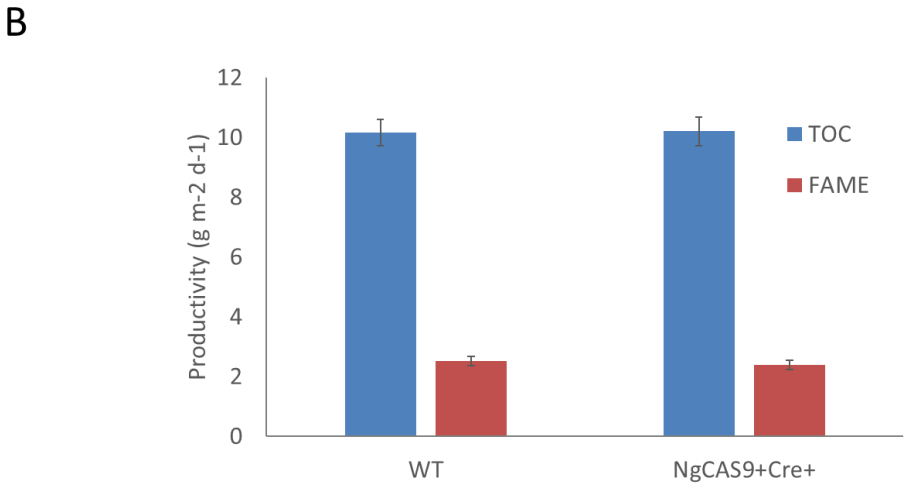

D
F12-NO3− isolated colonies
F12-NH4+
#1
#2
#3
E F G E F G E F G E F G
L
kb
-3.0
-1.0
-0.5

Figure S1. Molecular analysis of the progression from transformation to isolation of NgCAS9^{+}Cre^{+}. (A) Illustration of plasmid integration and subsequent excision of the floxed *GFP/Bsd* region, and expected amplicons from primer sets A-G (amplicons shown as red lines, not to scale). Expected amplicon sizes from the four main states of progression can be found in Table S3 (i.e. fully intact with no recombination, partially excised with circular recombination product remaining, fully excised with circular recombination product remaining, and fully excised with no circular recombination product remaining). Primer sequences can be found on Supplemental Table S1. (B) PCR assessment of Cre-mediated marker/reporter excision progress in NgCAS9^{+}Cre+ in CRM or CIM media. Primer set "A" spans the entire floxed region to detect the presence of intact floxed region (3.4 kb band), and/or a smaller 0.19 kb band indicating that recombination and excision have started to occur. Primer set "B" spans the 5' lox site, where an 0.85 kb band indicates intact floxed region and lack of a PCR amplicon indicates excision. Primer set "C" spans the 3' lox site where a 1.6 kb band indicates intact floxed region and lack of PCR amplicon indicates excision. Primer set "D" detects the presence of the circular recombination product; in the intact integrated state these primers are oriented away from each other, whereas in the circular recombination product they are oriented to produce an expected 1.3 kb band by PCR. Primer set "E" amplifies the Cre gene with an expected 1.4 kb band. Primer set "F" amplifies the Bsd gene with an expected band size of 0.4 kb. Primer set "G" amplifies the GFP gene with an expected 0.7kb gene. (C) Primer set "H" (Table S1) which produces a 1.3kb band for the excised form versus a 4.9kb band, included to provide better resolution of the excised form observed using primer set-A.(D) Analysis of single cell colony isolates from F12-NO_3^- culture screened with primers to detect Cre, Bsd, and GFP (Primer sets "E", "F", "G" respectively). Isolate #2 was carried forward as the final NgCas9^{+}Cre^{+} strain. The Lane labeled "L" in all gels refers to the NEB 2-Log DNA Ladder (0.1-10.0 kb).





C

Strain	σ_{PSII} (Å², 450nm)	Chl a/TOC (mg/g)
WT	454±11	64 ± 1
NgCAS9⁺Cre⁺	454±9	62 ± 2

Figure S2. qRT-PCR analysis of Cre expression and comparison of NgCAS9⁺Cre⁺ [22]and wild type (WT) strain performance under semi-continuous growth. (A) qRT-PCR results demonstrate that Cre expression is greatly repressed in the presence of NH_4^+. Expression levels in NH_4^+/NO_3^- are given a value of 1, while expression levels in NO_3^- are reported as relative to NH_4^+/NO_3^-. Expression levels were observed to increase by 40-fold in NO_3^-. (B) Semi-continuous growth mode TOC (blue) and FAME (red) areal productivities for wild type *Nannochloropsis* (WT) and NgCAS9⁺Cre⁺. (C) Chlorophyll content and functional absorption cross section of PSII (σ_{PSII}). Shown are the average and standard deviation of technical triplicates from cell culture duplicates (N = 6).

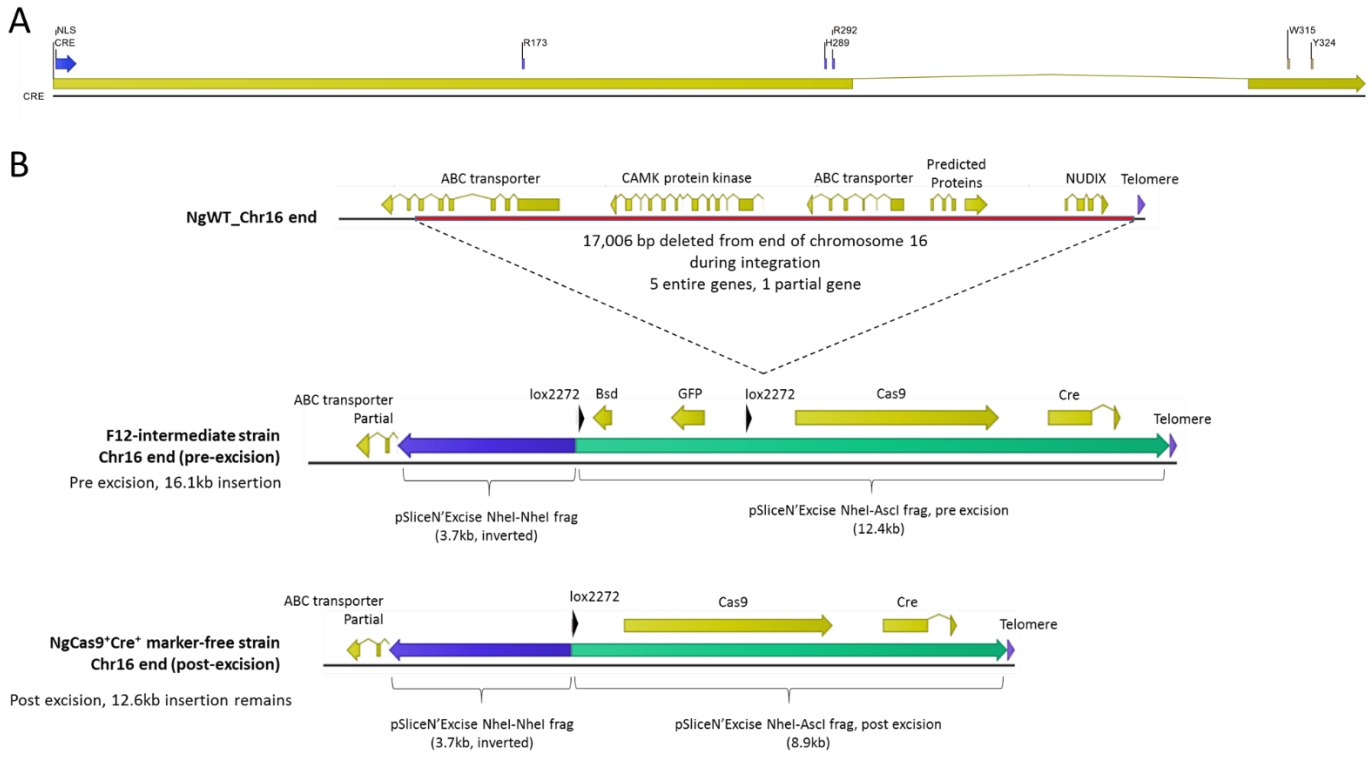
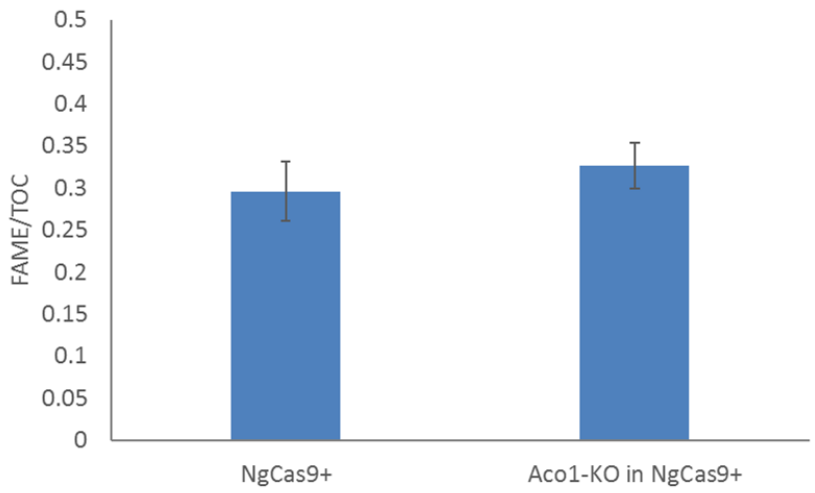


Figure S3. Architecture of the intronated recombinant Cre gene and locus of stable inserted Cas9 and Cre transgene in NgCAS9^{+}Cre^{+}. (A) Recombinant Cre gene has an N terminal NLS (SV40, shown in blue) and an intron from the native RPS4x gene transplanted between catalytic (R173, R289, and R292) and nucleophilic residues (W315 and Y324) to defunctionalize basal level CRE expression in *E.coli*. (B) Scheme depicting the integration of the Cas9 and Cre expression construct as deduced from PacBio genome sequencing of NgCas9^{+}Cre^{+}.

A



B

Strain	gene target	†Selectable marker	‡On-target marker insertion detected	Total transformants analyzed by PCR
NgCas9+	*Aco1*	HygR	90	160
NgCas9+Cre+	*Aco1*	Bsd-GFP	9	19

†Selectable markers co-transformed with the gRNA targeting Aco1 for Cas9-mediated insertional mutagenesis

‡A PCR band at the expected mass was observed (xx kb for HygR; yy kb for BSD-GFP).

Figure S4. Comparison of Cas9 mediated insertional mutagenesis in NgCas9+ (22) and NgCas9+Cre+ generated in this study. (A) Characterization of carbon partitioning to lipid (FAME/TOC) in an acyl-CoA oxidase gene knockout (*Aco1*) generated in the previously published *Nannochloropsis* Cas9 editor line (NgCas9+). Cultures were grown in biological duplicate in batch mode as previously described (22), and FAME (fatty acid methyl ester) and TOC (total organic carbon) measurements were taken daily for three days. Shown is the average and standard deviation of the FAME/TOC ratio used as a proximate indicator of partitioning of carbon to lipid. (B) Summary of results for targeting the *Aco1* gene in the original Cas9 editor line NgCas9+ and in the NgCas9+Cre+ line described in this study. The number of on-target insertions vs the total number of transformants analyzed by PCR is shown.

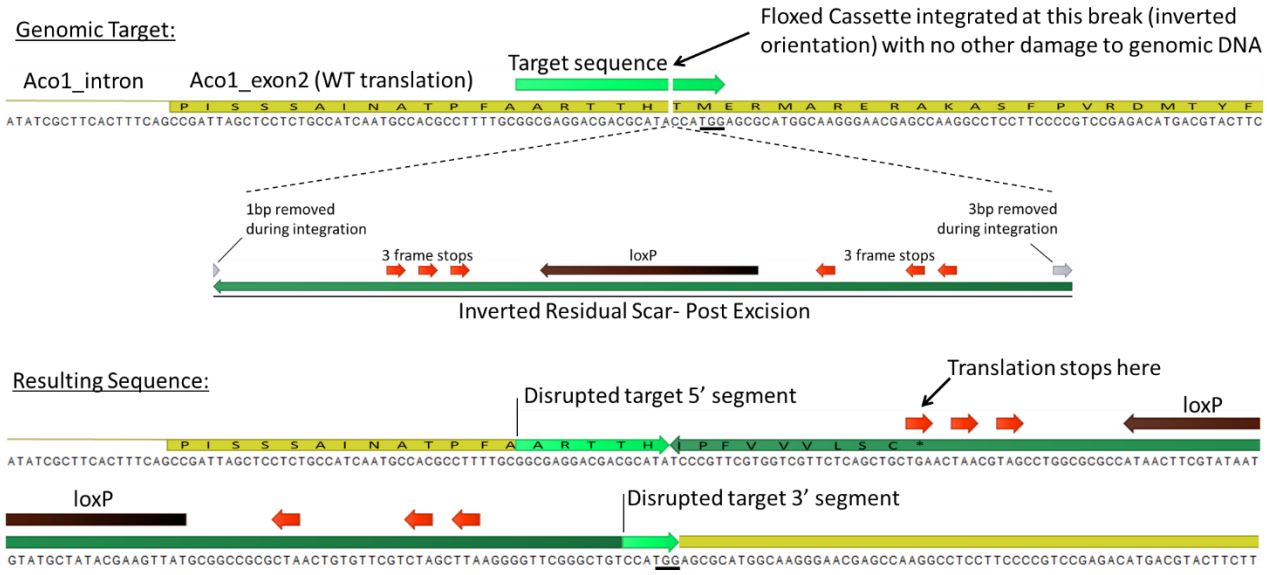


Figure S5. Diagram depicting PCR sequencing results at the *Aco1* knockout locus in isolates 1 to 3, where exon 2 was disrupted by the *lox* "scar" in an inverted orientation. The location and orientation of the insertion are indicated, as well as 1 and 3 bp deletions observed on the 5' and 3' ends of the integrated construct, respectively. All three isolates showed identical sequence results.

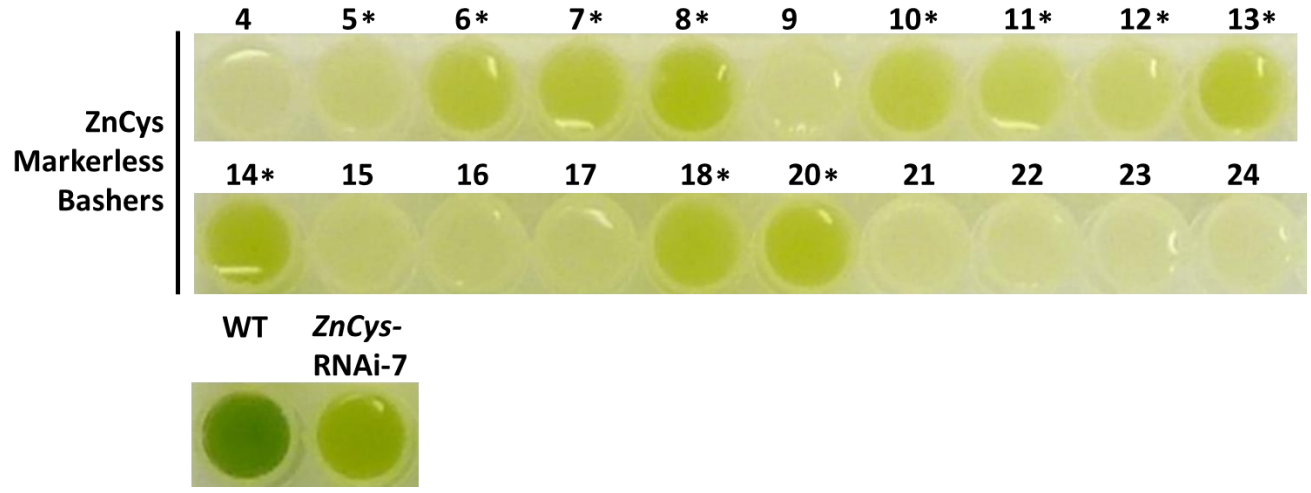


Figure S6. Range of pale phenotypes observed when *ZnCys* "5'-Bash" strains were cultured with medium containing NO_3^- as the sole N source, compared to wild type *Nannochloropsis gaditana.* A *ZnCys* RNAi strain from (22) was cultured in the same media, and strains exhibiting

visual pigmentation level comparable to the RNAi line were carried forward for isolation and genotyping (indicated here by asterisks).

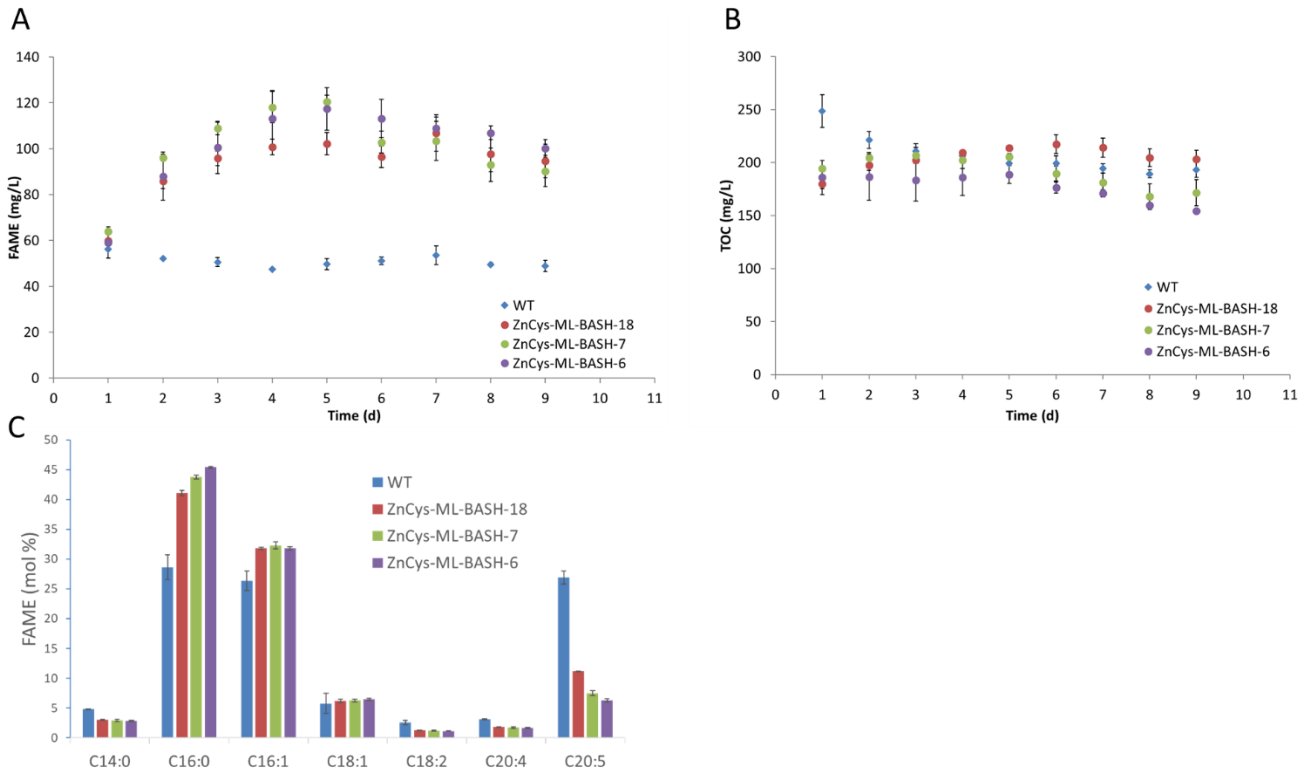


Figure S7. Volumetric FAME (A) and TOC (B) levels observed for wild-type and *Zncys*-ML-BASH lines under semi-continuous growth mode, where a 30 % daily dilution rate was applied. Shown are the average and standard deviation for biological triplicate cultures. Data from days 3 to 9 were used in the calculation of summary aerial productivities shown in Figure 3C. Total fatty acid profiles (C) were calculated from FAME data collected on day 5 of the run depicted in panels A and B.

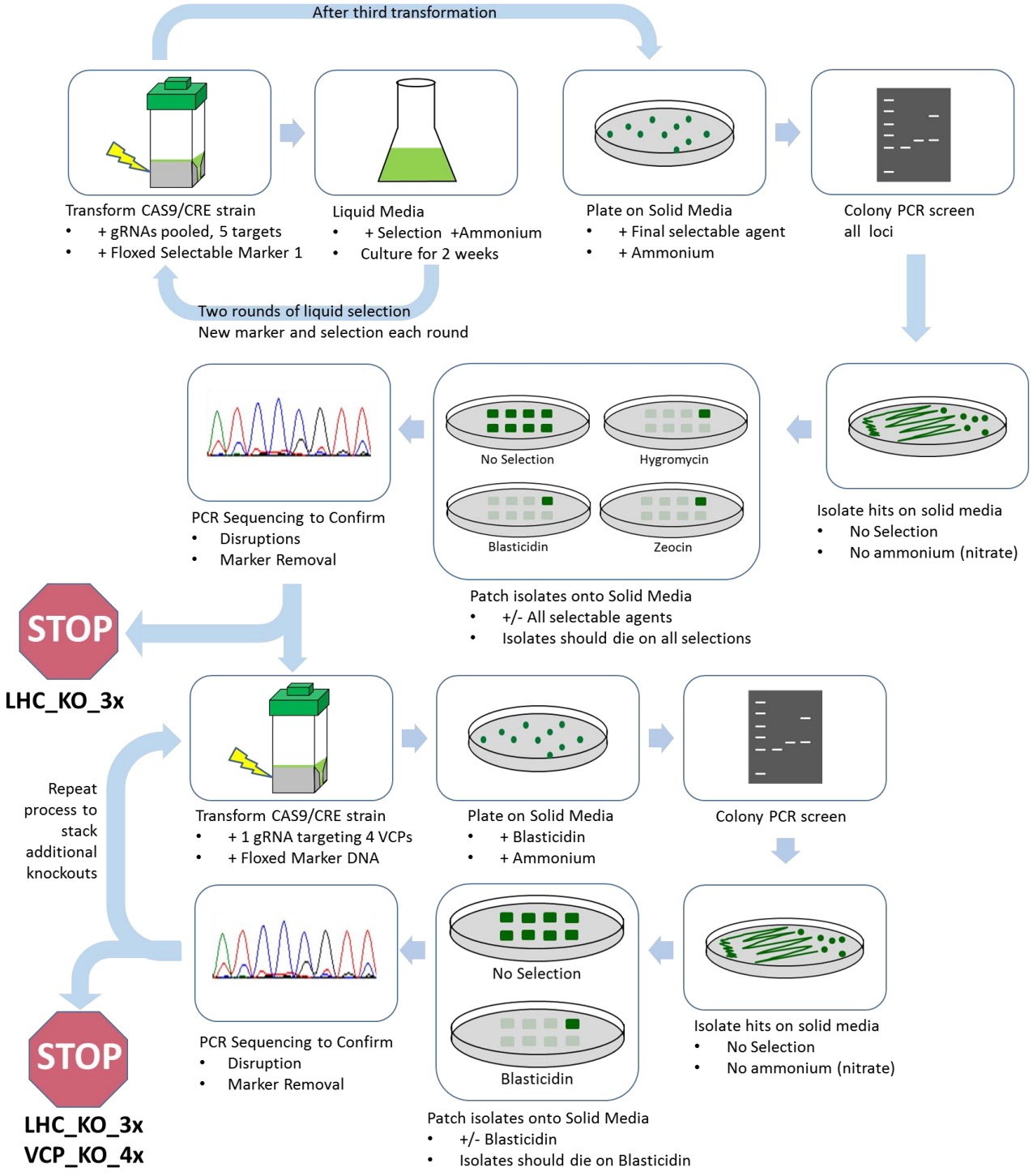


Figure S8. Rapid generation of strains harboring stacked knockout mutations in the light harvesting complex (LHC, non-VCP) and violaxanthin carrier protein (VCP) encoding gene families via a multiplexed iterative strategy in NgCAS9^{+}Cre^{+}. The diagram illustrates the method employed for the rapid stacking of knockouts, where briefly, the NgCAS9^{+}Cre^{+} strain was

transformed with 5 separate and unique gRNA molecules which target 5 highly expressed Light Harvesting Complex genes, and a floxed blasticidin resistance marker, and the transformed cells were inoculated into liquid media with blasticidin and NH_4^+ and cultured for 2 weeks. This culture was then transformed with the same 5 gRNAs again, but this time with a floxed hygromycin resistance marker, and the transformed cells were inoculated into liquid media with hygromycin and NH_4^+ and again cultured for 2 weeks. This culture was then transformed with the same 5 gRNAs, and a floxed zeocin resistance marker, and was plated onto solid media containing NH_4^+ and zeocin. Transformants were screened for inserts at the 5 loci by colony PCR with locus specific primers, and potential hits were streaked out for isolation on solid media with no selection and no NH_4^+. Isolated lines were analyzed by colony PCR at the 5 loci using locus specific primers which yielded one line, LHC-3x-, that was shown to have residual lox scars inserted in three of the five target loci and was sensitive to all antibiotics used. LHC-3x- was then transformed with a single gRNA which targets four separate violaxanthin-chlorophyll-a genes (VCP 1-4) and a floxed blasticidin resistance marker. Transformants were screened by colony PCR using unique locus specific primers. After cre-mediated marker excision and plating to isolation, one line, LHC_KO_3x/VCP_KO_4x, was found to have all four loci disrupted.

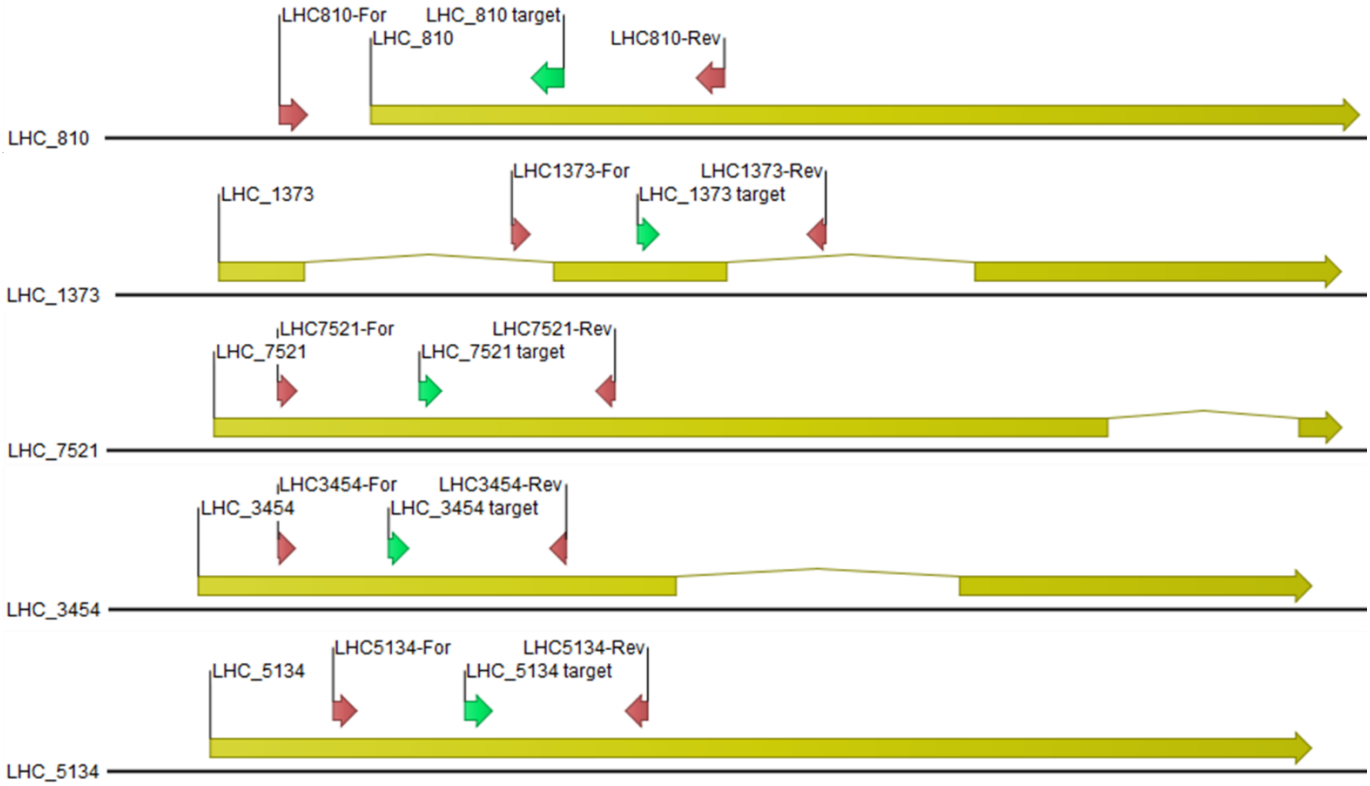


Figure S9. Five light harvesting complex (LHC, non-VCP) genes targeted for knockout and marker recycling. The coding sequences are shown in yellow, CRISPR targets in green, and screening primers in red.

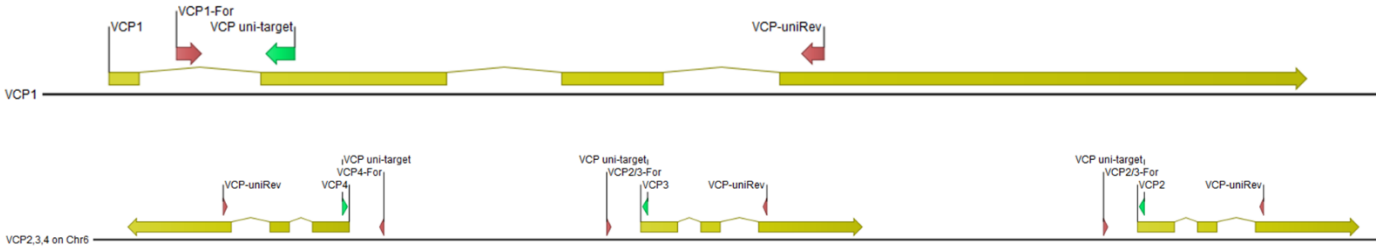


Figure S10. Light harvesting genes of the violaxanthin chlorophyll-a protein (VCP) family targeted for knockout and marker recycling. The coding sequences are shown in yellow, CRISPR targets in green, and screening primers in red.

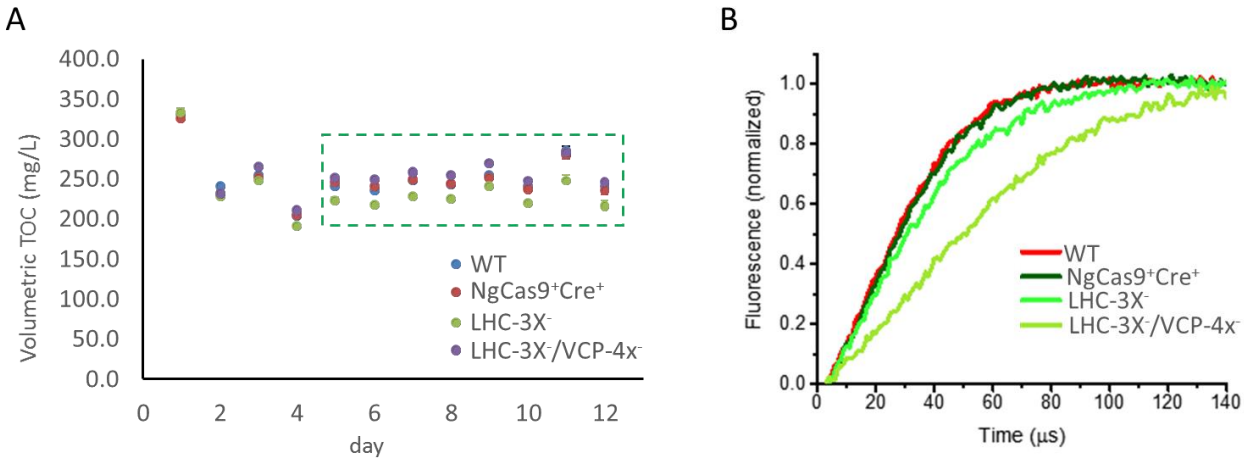


Figure S11. Characterization of LHC knockout strains for TOC productivity (A) and fluorescence induction (B) using the FIRe fluorimeter. Volumetric TOC for wild type (WT), NgCas9^{+}Cre^{+}, and the LHC mutants LHC-3X^{-} and LHC-3X^{-}/VCP-4X^{-} represent the average and standard deviation of cell culture triplicates for cultures diluted daily at 30 %; the data points within the dashed green box were used in determining areal TOC productivities summarized in Figure 4.

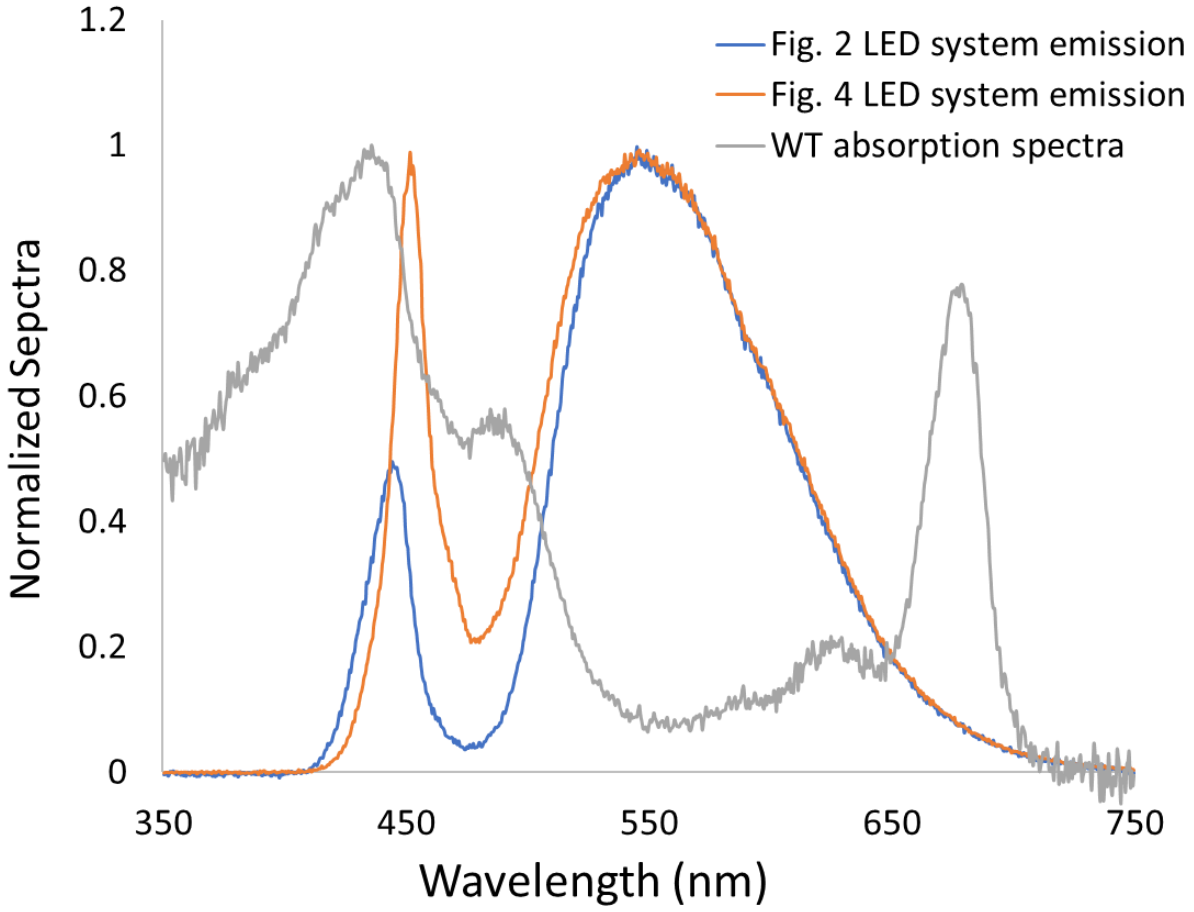


Figure S12. Peak normalized absorbance and emission spectra. Absorption spectra for wild type *N. gaditana* culture is shown in gray (measured using the Perkin Elmer Lambda 650 spectrophotometer as described in Methods). Emission spectra for the LED system used to assess strain productivity described in Figure 2 is shown in blue; Emission spectra for the updated LED system used to assess strain productivity in Figure 4 is shown in orange (both spectra were measured using Ocean Optics Jax Spectrometer). Spectra were normalized by setting the maxima to 1.

Supplemental tables:

Table S1. Primers used in this study.

I. Primers sequences used in analysis of the progression Cre-mediated marker removal leading to NgCAS9+Cre+.	
A/B/H-For	ctagcggccgcataac
A/C-Rev	AAACCATAATATGCCGTACCAACTG
F-For/B-Rev	atggccaagcctttgtcc
C-For/G-Rev	ttattcttcaccggcatctgc
D-For	CACAGCGGTTGGCATCAGGGAGTTGC
D-Rev	ggtgatgcggcactcgatctccatgg
E-For	ggtgcatcagaacttgcctgccttgcc
E-Rev	GCACCATTGCTCCCGTCTCGGAGTCC
F-Rev	tcaaccctcccagacgtagc
G-For	atgttggagagcgacgagagc
G-Rev	ttattcttcaccggcatctgc
H-Rev	CTTGAACTTCTTGGAAGGCACC
II. Screening Primers for CAS9 knockouts	
Aco1-For	TCAAAGATCATTTAGCAGAGACGGGGGCTGTGGCGCG
Aco1-Rev	AGTCGAGAGATGGTGCGTTCACGGATCTAGCCAGAG
ZnCys-5'Bash-For	TAGCAGAGCAGGCTCATCAC
ZnCys-5'Bash-Rev	GAATATGTGGTCTAGCTCGT
LHC810-For	CCCTTACTCCATCCCCAGAT
LHC810-Rev	ACTCGTCCCACTTGGTAGGC
LHC1373-For	TGGCTATGCTTCTCGTTCCT
LHC1373-Rev	ATTCCCCACACGACATCTCT
LHC7521-For	CTTCATGGGCAAGAACTTCG
LHC7521-Rev	GCGAAATCAGGGTTGGATAG
LHC3454-For	CTCTTCCCTCCCGAAAAACT
LHC3454-Rev	GCACCGACTTCGAGAACAC
LHC5134-For	TCTTCCTCCACCAGCTTTTT
LHC5134-Rev	GGAGGGTTGTTCGAGAAGC
VCP1-For	GTCTTTCTGGCTGGACTTGC
VCP-uniRev	GGTCACCAGGGTGTGGAG
VCP2/3-For	GACCCCGGAGGATCTTAGAC
VCP4-For	GGTCACCAGGGTGTGGAG

Table S2. Cas9 sites for loci targeted in this study.

CAS9 target sites	Sequence (PAM underlined)
Aco1 target (direct strand)	GGCGAGGACGACGCATACCATGG
ZnCys_5'UTR target (direct strand)	GGGACTGTCCCATTGTGCCGG
LHC_810 target (complementary strand)	GGGGGCTTCCAGGAAAGGGACGG
LHC_1373 target (direct strand)	GGCGAGCTTGCTGGGGATGTAGG

LHC_7521 target (direct strand)	GGTCTCTATCCCCAAACCCGCGG
LHC_3454 target (direct strand)	GGCCCTTCTCTGCTCCTCCGCGG
LHC_5134 target (direct strand)	GGCCCGTCGTGCCACCGGCGCGG
VCP uni-target (direct strand, present in all 4 VCP genes)	GGAGACGGTGAGCAGAGCGGCGG

Table S3. Description of expected PCR results for primer sets A-H.

Primer Set	Amplifies	Expected PCR bands (kb) from four states of progression			
		Fully Intact, no recombination	Partially excised, circular recombination product remains	Fully excised, circular recombination product remains	Fully excised, no circular recombination product remains
A	entire floxed region	3.7	0.185/3.7 (mix)	0.185	0.185
B	5' lox site	0.85	0.85 (faint)	no band	no band
C	3' lox site	1.6	1.6 (faint)	no band	no band
D	circular, excised recombination product	no band	1.3	1.3	no band
E	Cre only	1.4	1.4	1.4	1.4
F	Bsd only	0.4	0.4	0.4	no band
G	GFP only	0.7	0.7	0.7	no band
H	entire floxed region	4.9	1.3/4.9 (mix)	1.3	1.3

Table S4. Photophysiological phenotypes of LHC knockout lines grown under low incident irradiance as described in methods.

Data represent the average and standard deviation of biological duplicates.

Strain	σ_{PSII} (Å^2, 450nm)	σ_{PSII} (Å^2, 530nm)	N_{chl}/PSII	a_{chl} (m^2/g Chl)	Chl a/TOC (mg/g)
Wild Type	402 ± 2	163 ± 1	304 ± 9	4. 0± 0.1	79 ± 1
LHC-3X$^-$	374 ± 1	143 ± 1	255 ± 3	4.1 ± 0.1	66 ± 1
LHC-3X$^-$/VCP-4X$^-$	197 ± 2	92 ± 1	154 ± 6	4.9 ± 0.2	45 ± 1

Table S5. Description of DNA construct elements.

DNA element abbreviation	Function	Source/Description
BSD	Blasticidin resistance gene	codon optimized for N. gaditana
HygR	Hygromycin resistance gene	codon optimized for N. gaditana
Ble	Bleomycin/Zeocin resistance gene	codon optimized for N. gaditana
CAS9	CAS9 harboring N-terminal NLS and Flag epitope tag	codon optimized for N. gaditana
CRE	Cre recombinase harboring N-terminal NLS and intron from N.gaditana	codon optimized for N. gaditana
GFP	Turbo green fluorescent protein (Evrogen)	amplified from pTurboGFP-C (Evrogen)
TCT_P	Promoter	Amplified from N. gaditana gDNA
RPL24_P	Promoter	Amplified from N. gaditana gDNA
4AIII_P	Promoter	Amplified from N. gaditana gDNA
EIF3_P	Promoter	Amplified from N. gaditana gDNA
NIR_P	Promoter (NH_4^+ repressible)	Amplified from N. gaditana gDNA
EIF3_T	Terminator	Amplified from N. gaditana gDNA
FRD_T (bi-directional)	Terminator	Amplified from N. gaditana gDNA
GNPDA_T	Terminator	Amplified from N. gaditana gDNA
NIR_T	Terminator	Amplified from N. gaditana gDNA
loxP	lox site for recombination by Cre	synthesized
lox2272	lox site for recombination by Cre	synthesized
loxN	lox site for recombination by Cre	synthesized

.GB formatted sequence for pSliceN'Excise

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.GB formatted Sequence for loxP-BSD-GFP-loxP fragment

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DEFINITION  Complementary copy of pUC19.
KEYWORDS    ATCC.
COMMENT     This file is created by Vector NTI
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.GB formatted Sequence for loxN-HYGR-GFP-loxN fragment

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DEFINITION  Complementary copy of pUC19.
KEYWORDS    ATCC.
COMMENT     This file is created by Vector NTI
            http://www.invitrogen.com/
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.GB formatted Sequence for lox2272-BLE-GFP-lox2272 fragment

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DEFINITION  Complementary copy of pUC19.
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