Improving a *Synechocystis*-based photoautotrophic chassis through systematic genome mapping and validation of neutral sites

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Supplementary Table S1. Primers used for RT-PCR analysis

Primer name	Sequence 5' → 3'	Amplicon size (bp)
N1F	CATGGATTGAAGGAAGGG	100
N1R	TTTGGTGAACAATTAATGAAC	
N3F	GCAGTCCCTCCTCCAAAC	129
N3R	AAGCCGATATTCAGGTAAGATG	
N5F	GGAAGTGTTGTTGCTGTC	232
N5R	GTCCTTCGCCGTAGATTG	
N6F	AAGGTAATCAAGCAGAAATG	217
N6R	ATTGGTAACAGCACTTCC	
N7F	TGACTGTAAGCAATCTACC	500
N7R	ACTAACGATGGAATCTTGG	
N8F	AACTTAACTTCTATTCGTGAG	231
N8R	GAAACCTTGATAAGCAGTC	
N10F	GATATTGATGTGTATTGC	478
N10R	TTATTATCTTCTGTCTCC	
N11F	TGAATACCACATCTCCCATTGAC	200
N11R	TTTAACCTGCTGGCGTGAC	
N12F	TTCTCACCGCTACTGTTAC	227
N12R	CACTTCTCGCACTAATTCG	
N13F	CCCAAATGATGACCGAAG	216
N13R	CTAGTAACTGCTGTTCCTC	
N14F	AAGTGTCCAAAGCCAAAC	270
N14R	TCAACAATGCCATCTTCC	
N15F	TTAATTGCCACTGCCTTAC	293
N15R	TAGAGACAGCGGATATGC	
N16F	TGATGTGGACGAGGATAC	335
N16R	GGGCTAAGGGAATATGGG	

Supplementary Table S2. Other primers used in this work

Primer name	Sequence 5' → 3'[a]	Purpose
Km.KmScFwd	CTGACCCCGGGTGAATGTCAGCTACTGG	Selection cassettes amplification
KmRev	CAAACCCGGGCGATTTACTTTTCGACCTC	
KmScRev	ACAGACCCGGGCAAGCGGATGGCTGATG	
N5.5O[b]	GCGGCCTCGAGTGGGGGCATCTGCTAGGCAATATTTG	Amplification of the DNA fragments flanking the neutral sites
N5.5I	GATTACACCCGGGTGTAATCGGTTGATTATTTCAGTGGCCCGGCCG ATGGATAATTAC	
N5.3O[b]	GTCCAAATCAGCATTGCTCTGCCAGGTGAGAC	
N5.3I	GATTACACCCGGGTGTAATCCTGAAGCCTTGACAATCCCCGTTGGT GATTTATACTTAG	
N8.5O[b]	GAACACTCGAGTGCCAGCAACGACAATG	
N8.5I	GATTACACCCGGGTGTAATCCTTCATTCCCCTATTGTTATTAATAGTG CTC	
N8.3O[b]	GATGACCGCTGGCGGAGTTTAGTCCAG	
N8.3I	GATTACACCCGGGTGTAATCTTGTCACCAATTTTTGTAGGGATGTTG GGCTAAATTG	
N10.5O[b]	GATAACTCGAGTCGCCCGGTGGATTTAATGC	
N10.5I	GATTACACCCGGGTGTAATCCCCATACTCAGCCTTCTAATGCTGAGA GAATG	
N10.3O[b]	GGACTGACCCAAGATACAGTGGTAG	
N10.3I	GATTACACCCGGGTGTAATCCGAGCGCAAACTACAATGCGCTTCGT TTGC	
N15.5O[b]	GGTTCTCGAGCCGCTGAATTTAGTCAACATCG	
N15.5I	GATTACACCCGGGTGTAATCCATTGGGCACGAGAGTTAGTAAGGCA GTG	
N15.3O[b]	CTAAACTTACGGCATTGGCATCAACGGGAG	
N15.3I	GATTACACCCGGGTGTAATCTCTTTACAATGGCCAGGTCTTTAGGGA GCGGTGAC	
N16.5O[b]	GAACTCGAGTAGTAACCACAGGCTTTTG	
N16.5I	GATTACACCCGGGTGTAATCGCTGGAGGCGAACTGGGTGAGAACCA T	
N16.3O[b]	GTGAGCTTGATGGTGATGGTGGGTAAAG	
N16.3I	GATTACACCCGGGTGTAATCTGCTGGCTTTGTTGCCCTGTCAACCAA AGTTC	
N5_SDM	CGAGGCGATCGCCCAGTTGGAAGAATTGGCC	Site directed mutagenesis
N10_SDM	CTAAAAAGACAAGTCTGTGGCTAGTTACTATGACGAGGC	
N5.FO	TCCTGGTAACTCACGCTATC	Mutants confirmation by PCR
N5.FI	AGCCGATCCAGGGAAGTGTTGTTG	
N5.RI	CCATCGTCCTTCGCCGTAGATTGTG	
N8.FO	CCCAGTTAAACTGCGAAAGG	
N8.FI	TCGCCAAGCTTTCAGAAC	
N8.RI	CAAACTCCAGCCGATAAC	
N10.FO	CCGGTTGCCCTTATCGGAACCGATG	
N10.FI	GCTATGGCGTCACTTGTAGC	
N10.RI	TTTGCGACCCATCGGATTGC	
N15.FO	CTCCAAGGCGACTACCTTTC	
N15.FI	CCCAGTGGGAATGCGATCAG	

N15.RI	TAGGAGGGCGATCACCGAAG	
N16.FO	ACCCATTTCTTGGGTGTAGG	Mutants confirmation by PCR
N16.FI	GGCCTTGGTTGCCCTGACTGATGTG	
N16.RI	GACCGATCGCCGCAGTAGTTCTTGG	
GFP.F	TCTTGTTGAATTAGATGGTG	RT-PCR/RT-qPCR
GFP.R	TGTGAGTTATAGTTGTATTCC	

[a]Restriction sites underlined
[b]Primers used to confirm genomic integrations and mutants full segregation.

Supplementary Table S3. List of the putative neutral sites identified

Site name	ORF ID	Chromosome position	Orientation[a]	Length of the putatively encoded protein (amino acids)
N1	*ssl0606*	2441925 - 2442083	c	52
N2	*slr0368*	2365848 - 2366120	d	90
N3	*ssl0318*	2302457 - 2302645	c	62
N4	*ssr0680*	2684766 - 2684990	d	74
N5	*sll1476*	3398409 - 3398717	c	102
N6	*slr1869*	1212609 - 1213358	d	249
N7	*sll0494*	3224429 - 3225334	c	301
N8	*slr0573*	2816517 - 2816960	d	147
N9	*sll0181*	2737929 - 2738552	c	207
N10	*slr1396*	707437 - 708054	d	205
N11	*ssr1038*	2950107 - 2950343	d	78
N12	*ssl3615*	776058 - 776342	c	94
N13	*slr0587*	3536090 - 3536422	d	110
N14	*sll0167*	2317536 - 2318030	c	164
N15	*slr0271*	1524568 - 1525086	d	172
N16	*slr0397*	2146801 - 2147376	d	191

[a] d – direct sequence, c – complement sequence.

Supplementary Table S4. ANOVA analysis of *Synechocystis* wild-type growth compared to the SN*n*K mutants. *P*-values of up to second order interactions are shown.

	Mutants				
Parameters	**SN5K**	**SN8K**	**SN10K**	**SN15K**	**SN16K**
Light	$1.61x10^{-38}$	$3.47x10^{-37}$	$8.70x10^{-34}$	$1.58x10^{-33}$	$5.28x10^{-39}$
Glucose	$1.49x10^{-11}$	$3.62x10^{-22}$	$8.48x10^{-19}$	$2.54x10^{-12}$	$3.50x10^{-25}$
Mutation[a]	$4.58x10^{-15}$	$1.24x10^{-04}$	$8.65x10^{-03}$	$3.37x10^{-01}$	$3.48x10^{-01}$
Light + Glucose	$2.57x10^{-10}$	$1.36x10^{-14}$	$3.62x10^{-18}$	$6.46x10^{-20}$	$9.58x10^{-16}$
Light + Mutation	$1.74x10^{-03}$	$4.51x10^{-04}$	$1.04x10^{-01}$	$5.27x10^{-01}$	$7.76x10^{-02}$
Glucose + Mutation	$1.74x10^{-07}$	$1.00x10^{-01}$	$1.20x10^{-01}$	$1.44x10^{-04}$	$1.30x10^{-01}$

[a]The statistical analysis of the effect produced by each mutation introduced is highlighted in grey.

Supplementary Table S5. iTRAQ samples labelling and experimental design

Label	iTRAQ a	iTRAQ b
113	WT1[a]	
114	WT2[a]	
115	SN15K.Cgfp1	SN5K.Cgfp1
116	SN15K.Cgfp2	SN5K.Cgfp2
117	SN16K.Cgfp1	SN8K.Cgfp1
118	SN16K.Cgfp2	SN8K.Cgfp2
119	SN15K.gfp1	SN10K.Cgfp1
121	SN15K.gfp2	SN10K.Cgfp2

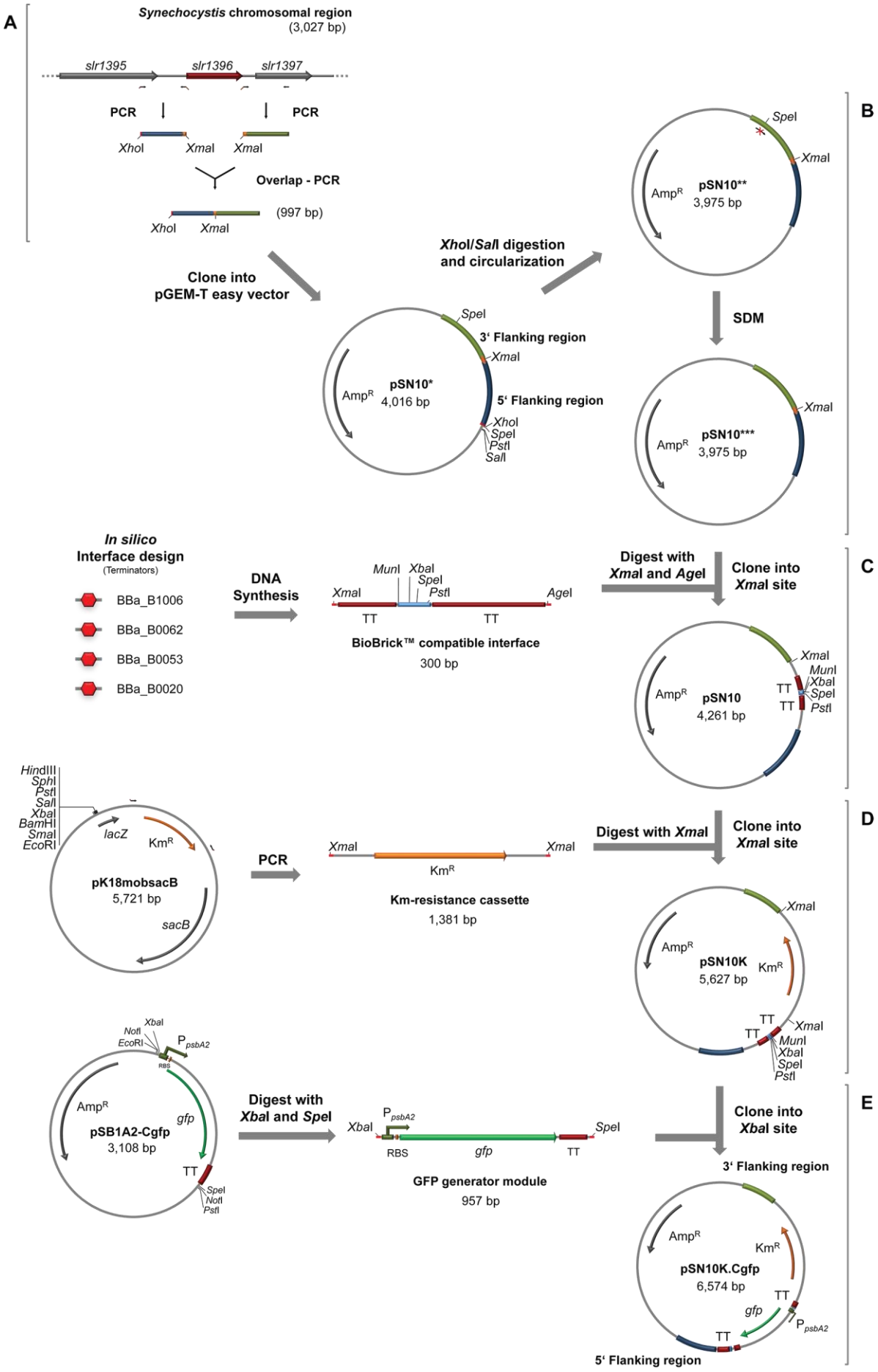
[a]Samples common to both iTRAQ experiments

Supplementary Table S6. List of plasmids constructed in this work

Plasmid name	Description
pSN5	pGEM-T easy vector containing the BioBrick compatible interface flanked by the two regions for double homologous recombination on neutral site N5.
pSN8	pGEM-T easy vector containing the BioBrick compatible interface flanked by the two regions for double homologous recombination on neutral site N8.
pSN10	pGEM-T easy vector containing the BioBrick compatible interface flanked by the two regions for double homologous recombination on neutral site N10.
pSN15	pGEM-T easy vector containing the BioBrick compatible interface flanked by the two regions for double homologous recombination on neutral site N15.
pSN16	pGEM-T easy vector containing the BioBrick compatible interface flanked by the two regions for double homologous recombination on neutral site N16.
pSN5K	pSN5 plasmid with the kanamycin resistance cassette cloned upstream of the BioBrick compatible interface.
pSN8K	pSN8 plasmid with the kanamycin resistance cassette cloned upstream of the BioBrick compatible interface.
pSN10K	pSN10 plasmid with the kanamycin resistance cassette cloned upstream of the BioBrick compatible interface.
pSN15K	pSN15 plasmid with the kanamycin resistance cassette cloned upstream of the BioBrick compatible interface.
pSN16K	pSN16 plasmid with the kanamycin resistance cassette cloned upstream of the BioBrick compatible interface.
pSN5KS	pSN5 plasmid with the kanamycin resistance/sucrose sensitivity cassette cloned upstream of the BioBrick compatible interface.
pSN8KS	pSN8 plasmid with the kanamycin resistance/sucrose sensitivity cassette cloned upstream of the BioBrick compatible interface.
pSN10KS	pSN10 plasmid with the kanamycin resistance/sucrose sensitivity cassette cloned upstream of the BioBrick compatible interface.
pSN15KS	pSN15 plasmid with the kanamycin resistance/sucrose sensitivity cassette cloned upstream of the BioBrick compatible interface.
pSN16KS	pSN16 plasmid with the kanamycin resistance/sucrose sensitivity cassette cloned upstream of the BioBrick compatible interface.
pSN5K.gfp	pSN5K plasmid with the GFP generator BioBrick BBa_E0240 cloned in the BioBrick compatible interface.
pSN8K.gfp	pSN8K plasmid with the GFP generator BioBrick BBa_E0240 cloned in the BioBrick compatible interface.
pSN10K.gfp	pSN10K plasmid with the GFP generator BioBrick BBa_E0240 cloned in the BioBrick compatible interface.
pSN15K.gfp	pSN15K plasmid with the GFP generator BioBrick BBa_E0240 cloned in the BioBrick compatible interface.
pSN16K.gfp	pSN16K plasmid with the GFP generator BioBrick BBa_E0240 cloned in the BioBrick compatible interface.
pSN5K.Cgfp	pSN5K plasmid with the GFP generator BioBrick BBa_E0240 under the control of a constitutive promoter (P_{psbA2*}), cloned in the BioBrick compatible interface.
pSN8K.Cgfp	pSN8K plasmid with the GFP generator BioBrick BBa_E0240 under the control of a constitutive promoter (P_{psbA2*}), cloned in the BioBrick compatible interface.
pSN10K.Cgfp	pSN10K plasmid with the GFP generator BioBrick BBa_E0240 under the control of a constitutive promoter (P_{psbA2*}), cloned in the BioBrick compatible interface.
pSN15K.Cgfp	pSN15K plasmid with the GFP generator BioBrick BBa_E0240 under the control of a constitutive promoter (P_{psbA2*}), cloned in the BioBrick compatible interface.
pSN16K.Cgfp	pSN16K plasmid with the GFP generator BioBrick BBa_E0240 under the control of a constitutive promoter (P_{psbA2*}), cloned in the BioBrick compatible interface.

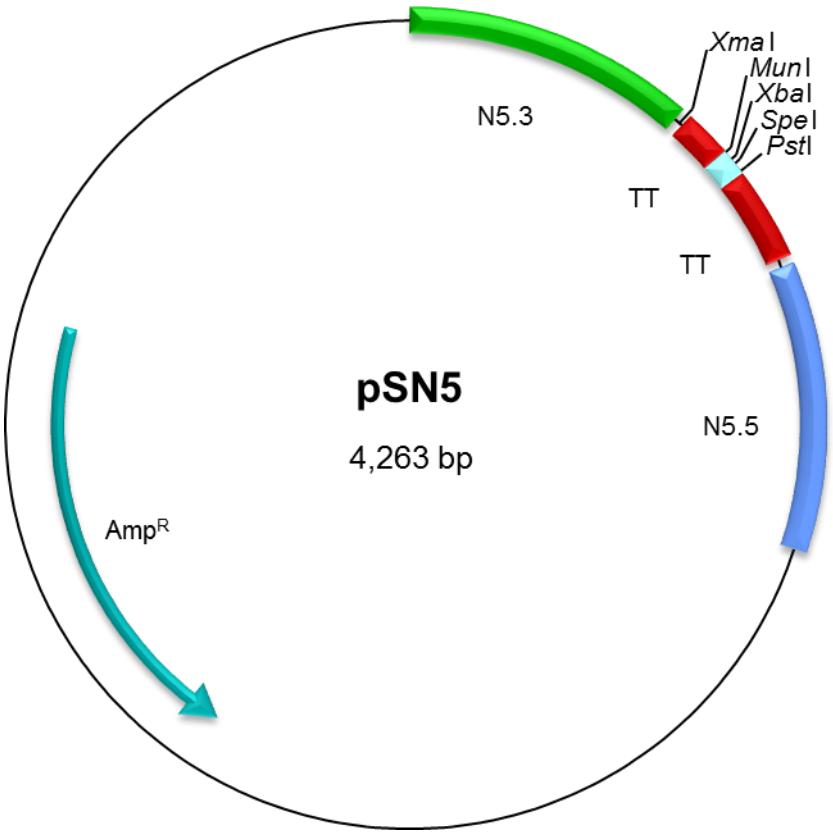
Supplementary Table S7. List of *Synechocystis* mutants constructed in this work

Mutant name	Description
SN5K	*Synechocystis* mutant with replacement of the neutral site N5 by a kanamycin resistance cassette.
SN8K	*Synechocystis* mutant with replacement of the neutral site N8 by a kanamycin resistance cassette.
SN10K	*Synechocystis* mutant with replacement of the neutral site N10 by a kanamycin resistance cassette.
SN15K	*Synechocystis* mutant with replacement of the neutral site N15 by a kanamycin resistance cassette.
SN16K	*Synechocystis* mutant with replacement of the neutral site N16 by a kanamycin resistance cassette.
SN5KS	*Synechocystis* mutant with replacement of the neutral site N5 by a kanamycin resistance/sucrose sensitivity cassette.
SN8KS	*Synechocystis* mutant with replacement of the neutral site N8 by a kanamycin resistance/sucrose sensitivity cassette.
SN10KS	*Synechocystis* mutant with replacement of the neutral site N10 by a kanamycin resistance/sucrose sensitivity cassette.
SN15KS	*Synechocystis* mutant with replacement of the neutral site N15 by a kanamycin resistance/sucrose sensitivity cassette.
SN16KS	*Synechocystis* mutant with replacement of the neutral site N16 by a kanamycin resistance/sucrose sensitivity cassette.
SN5K.gfp	*Synechocystis* mutant with replacement of the neutral site N5 by a kanamycin resistance cassette and the promoterless GFP encoding sequence.
SN8K.gfp	*Synechocystis* mutant with replacement of the neutral site N8 by a kanamycin resistance cassette and the promoterless GFP encoding sequence.
SN10K.gfp	*Synechocystis* mutant with replacement of the neutral site N10 by a kanamycin resistance cassette and the promoterless GFP encoding sequence.
SN15K.gfp	*Synechocystis* mutant with replacement of the neutral site N15 by a kanamycin resistance cassette and the promoterless GFP encoding sequence.
SN16K.gfp	*Synechocystis* mutant with replacement of the neutral site N16 by a kanamycin resistance cassette and the promoterless GFP encoding sequence.
SN5K.Cgfp	*Synechocystis* mutant with replacement of the neutral site N5 by a kanamycin resistance cassette and the GFP encoding sequence under the control of a constitutive promoter (P_{psbA2*}).
SN8K.Cgfp	*Synechocystis* mutant with replacement of the neutral site N8 by a kanamycin resistance cassette and the GFP encoding sequence under the control of a constitutive promoter (P_{psbA2*}).
SN10K.Cgfp	*Synechocystis* mutant with replacement of the neutral site N10 by a kanamycin resistance cassette and the GFP encoding sequence under the control of a constitutive promoter (P_{psbA2*}).
SN15K.Cgfp	*Synechocystis* mutant with replacement of the neutral site N15 by a kanamycin resistance cassette and the GFP encoding sequence under the control of a constitutive promoter (P_{psbA2*}).
SN16K.Cgfp	*Synechocystis* mutant with replacement of the neutral site N16 by a kanamycin resistance cassette and the GFP encoding sequence under the control of a constitutive promoter (P_{psbA2*}).


A
Synechocystis chromosomal region
(3,027 bp)
slr1395
slr1396
slr1397
PCR
PCR
XhoI
XmaI
XmaI
Overlap - PCR
(997 bp)
XhoI
XmaI
Clone into
pGEM-T easy vector
XhoI/SalI digestion
and circularization
B
SpeI
XmaI
AmpR
pSN10**
3,975 bp
SDM
SpeI
3' Flanking region
XmaI
AmpR
pSN10*
4,016 bp
5' Flanking region
XhoI
SpeI
PstI
SalI
XmaI
AmpR
pSN10***
3,975 bp
In silico
Interface design
(Terminators)
BBa_B1006
BBa_B0062
BBa_B0053
BBa_B0020
DNA
Synthesis
MunI
XbaI
SpeI
PstI
XmaI
AgeI
TT
TT
BioBrick™ compatible interface
300 bp
Digest with
XmaI and AgeI
Clone into
XmaI site
C
XmaI
MunI
XbaI
SpeI
PstI
AmpR
pSN10
4,261 bp
TT
TT
HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
EcoRI
lacZ
KmR
pK18mobsacB
5,721 bp
sacB
PCR
XmaI
XmaI
KmR
Km-resistance cassette
1,381 bp
Digest with XmaI
Clone into
XmaI site
D
XmaI
AmpR
pSN10K
5,627 bp
KmR
TT
TT
XmaI
MunI
XbaI
SpeI
PstI
NotI
EcoRI
XbaI
PpsbA2
RBS
AmpR
gfp
pSB1A2-Cgfp
3,108 bp
TT
SpeI
NotI
PstI
Digest with
XbaI and SpeI
XbaI
PpsbA2
RBS
gfp
TT
SpeI
GFP generator module
957 bp
Clone into
XbaI site
E
3' Flanking region
AmpR
KmR
pSN10K.Cgfp
6,574 bp
TT
gfp
PpsbA2
TT
5' Flanking region

Supplementary Figure S1. Schematic representation of pSN10K.Cgfp construction. Homologous regions flanking the putative neutral site were amplified by PCR (primers indicated by arrows) and fused by overlap-PCR (**A**). The fragment containing the two flanking regions was cloned into the pGEM-T easy vector; the restriction sites incompatible with the BioBrick standard RFC[10] were removed by digestion with *Xho*I and *Sal*I and vector re-circularization, and by site directed mutagenesis (SDM, mutagenic primers indicated by a crossed arrow) (**B**). The BioBrick-compatible cloning interface, flanked by two double BioBrick transcription terminators (TT), was synthesized and cloned into the *Xma*I site of the vector originating the pSN10 plasmid (**C**). A selection cassette conferring resistance to kanamycin was amplified by PCR (primers indicated by arrows) from the plasmid pK18mobsacB and cloned into the *Xma*I site (pSN10K) (**D**). Subsequently, the module containing the GFP encoding sequence under the control of the minimal *psbA2* promoter (P_{psbA2^*}) was excised from the BioBrick vector pSB1A2-Cgfp and cloned into the cloning interface, originating the integrative plasmid pSN10K.Cgfp (**E**). For details see Material and Methods.

A



B

>pSN5
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CTGTGCTCGAAAATCGGGGCTACATTGCTTCTTGCTACACCTGTGGCGACCAACTCAACAGCGCCTCCTTCATGGTGAGCTTGGGGGAAAATCA
TCTGATCCGCTTTTTGGTATCGGACTACGGCATCACCTGGACAGAAATGCGGGATGACCGAGAATTAATGAAATTAGAAGGAGCCGAGGCGATC
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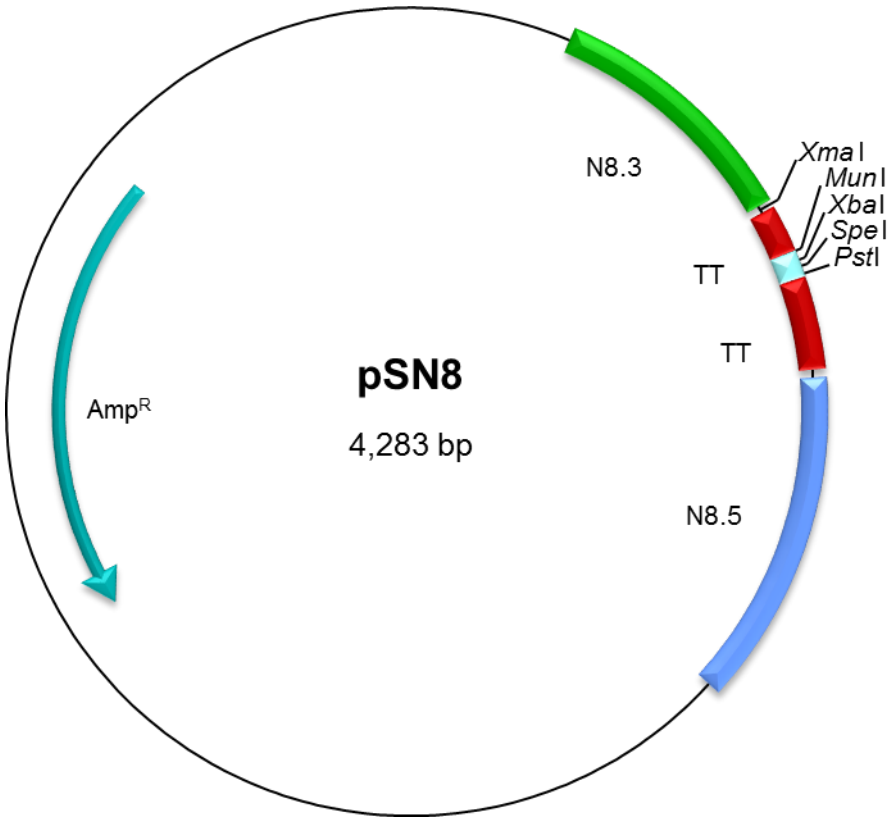
CGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAA
CGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGC
TCCCGGCCGCCATGGCGGCCGCGGGAATTCGAT

Supplementary Figure S2. Plasmid pSN5. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N5.3 – 3' homologous region for site N5; N5.5 – 5' homologous region for site N5; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance.

A



B

>pSN8
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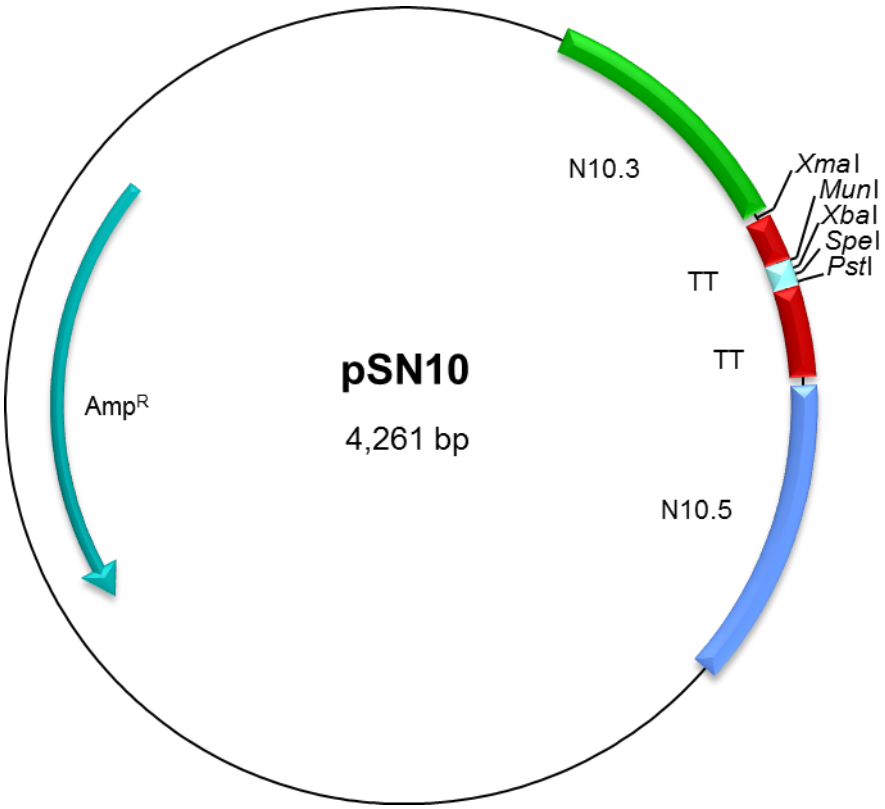
Supplementary Figure S3. Plasmid pSN8. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N8.3 – 3' homologous region for site N8; N8.5 – 5' homologous region for site N8; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance.

A



B

>pSN10
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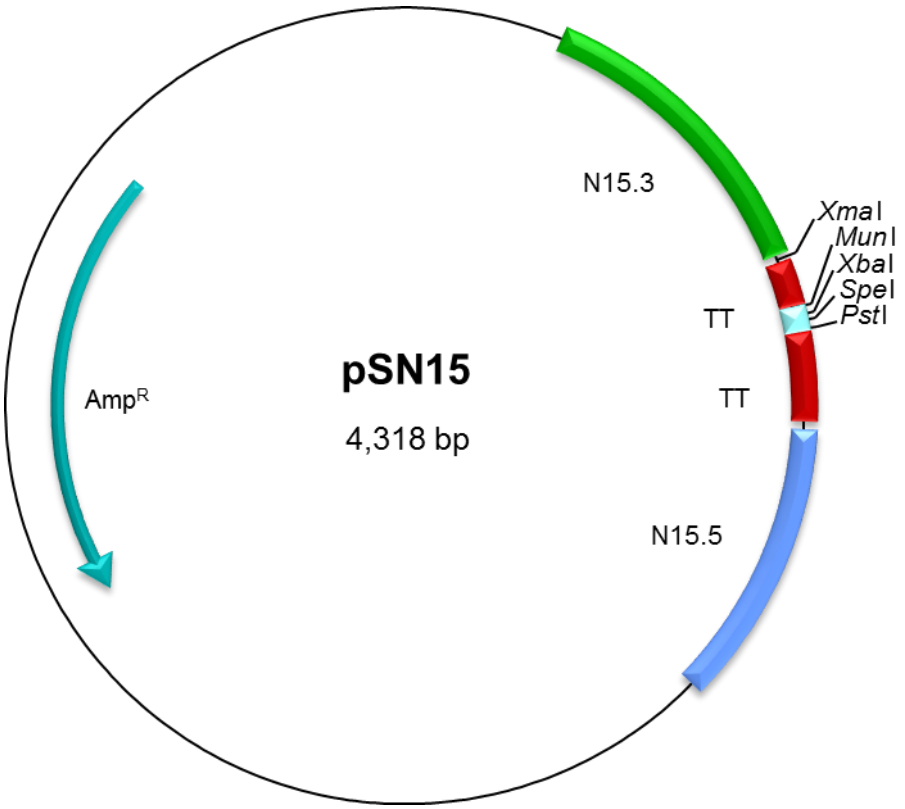
Supplementary Figure S4. Plasmid pSN10. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N10.3 – 3’ homologous region for site N10; N10.5 – 5’ homologous region for site N10; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance.

A



B

>pSN15
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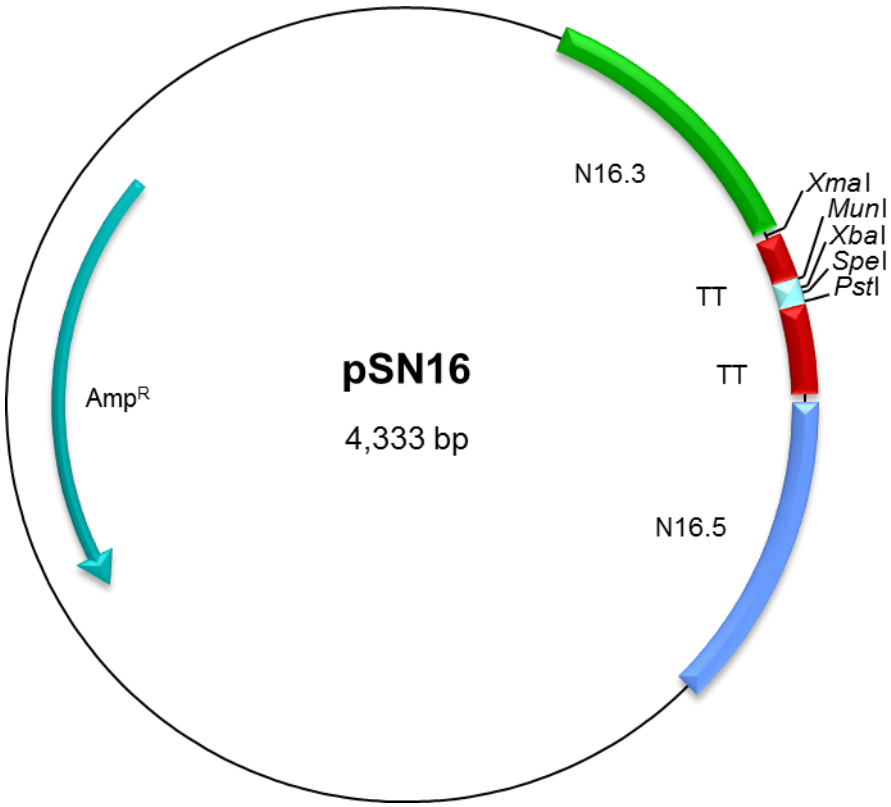
Supplementary Figure S5. Plasmid pSN15. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N15.3 – 3' homologous region for site N15; N15.5 – 5' homologous region for site N15; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance.

A



B

>pSN16
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Supplementary Figure S6. Plasmid pSN16. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N16.3 – 3' homologous region for site N16; N16.5 – 5' homologous region for site N16; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance.

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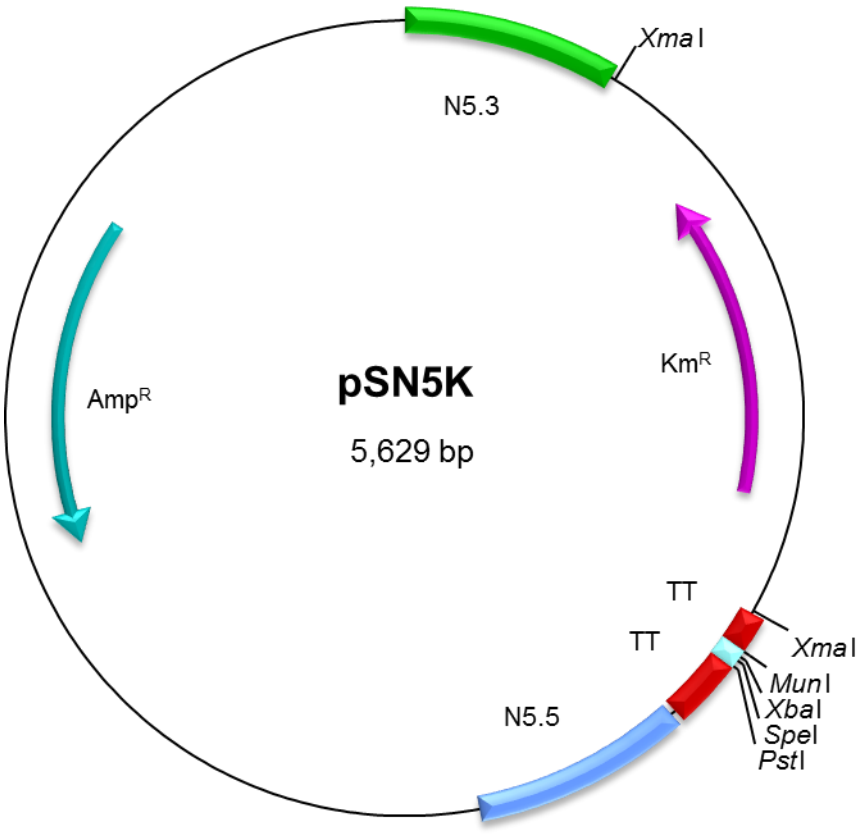

B

>pSN5K

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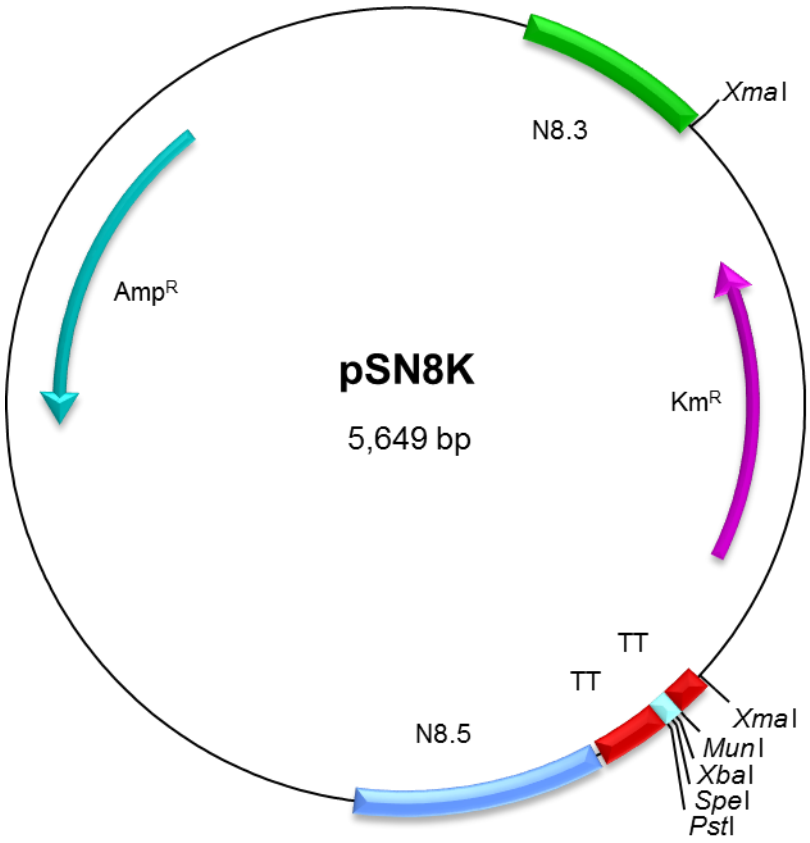
Supplementary Figure S7. Plasmid pSN5K. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N5.3 – 3' homologous region for site N5; N5.5 – 5' homologous region for site N5; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance.

A



B

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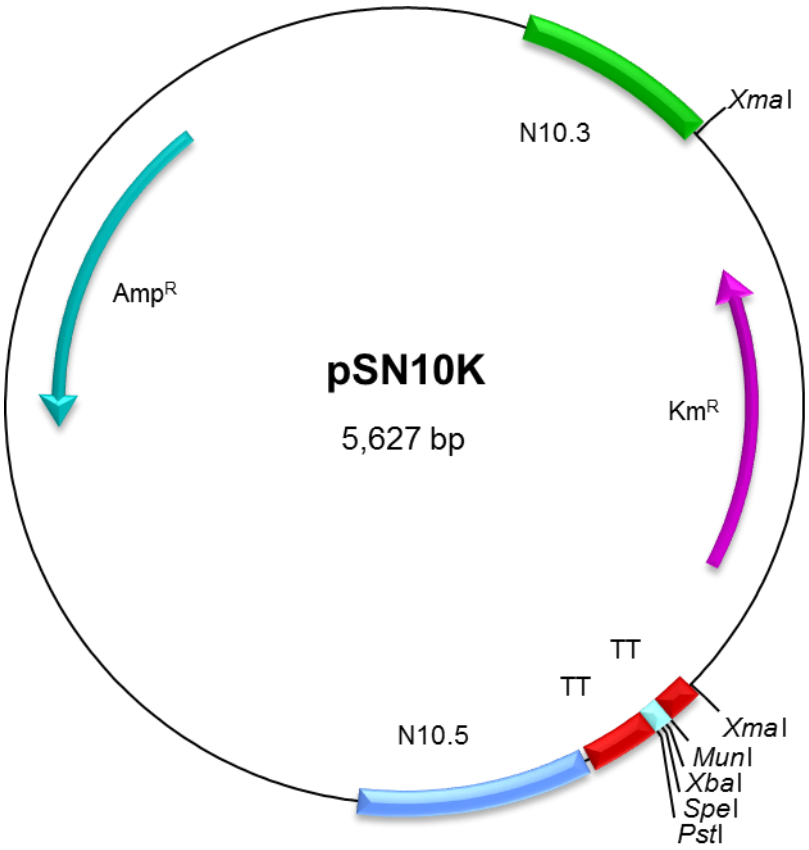
Supplementary Figure S8. Plasmid pSN8K. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N8.3 – 3' homologous region for site N8; N8.5 – 5' homologous region for site N8; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance.

A



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Supplementary Figure S9. Plasmid pSN10K. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N10.3 – 3' homologous region for site N10; N10.5 – 5' homologous region for site N10; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance.

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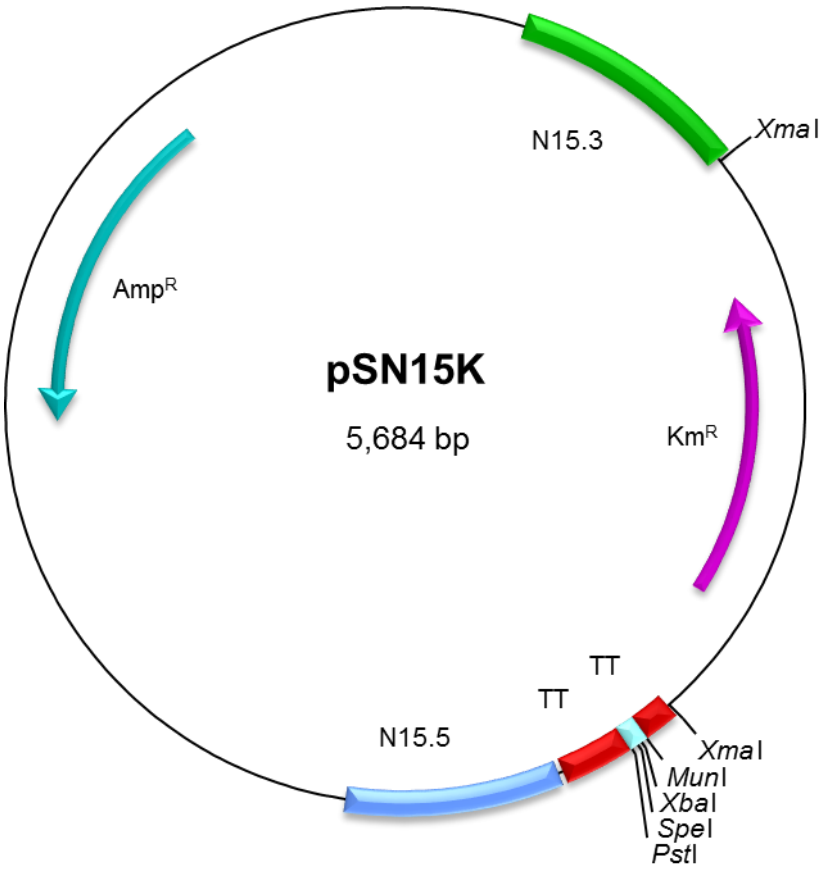

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>pSN15K
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Supplementary Figure S10. Plasmid pSN15K. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N15.3 – 3' homologous region for site N15; N15.5 – 5' homologous region for site N15; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance.

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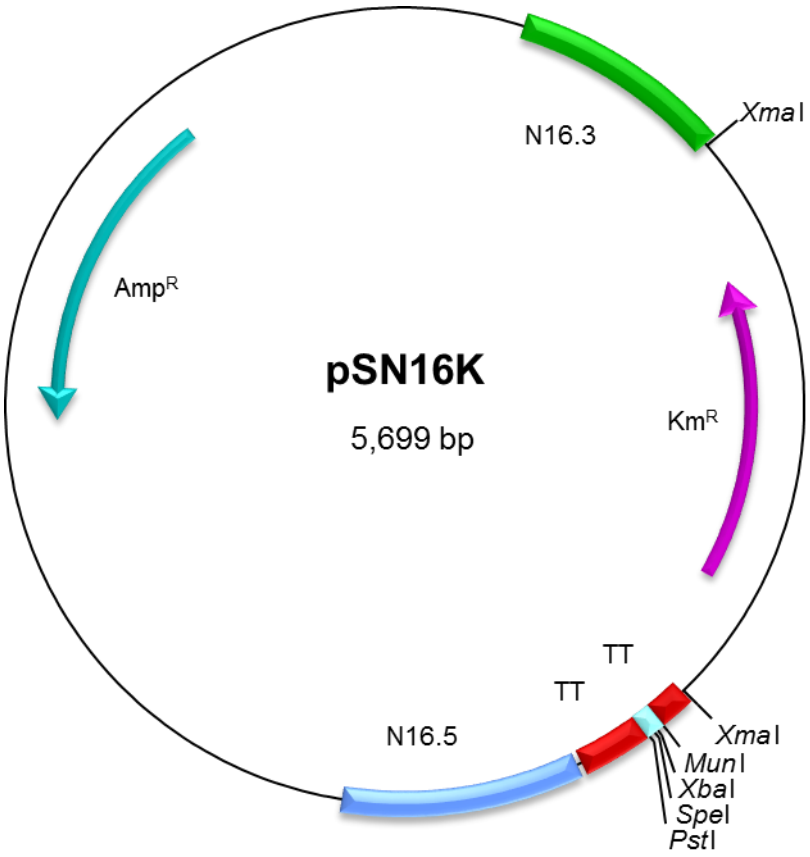

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>pSN16K

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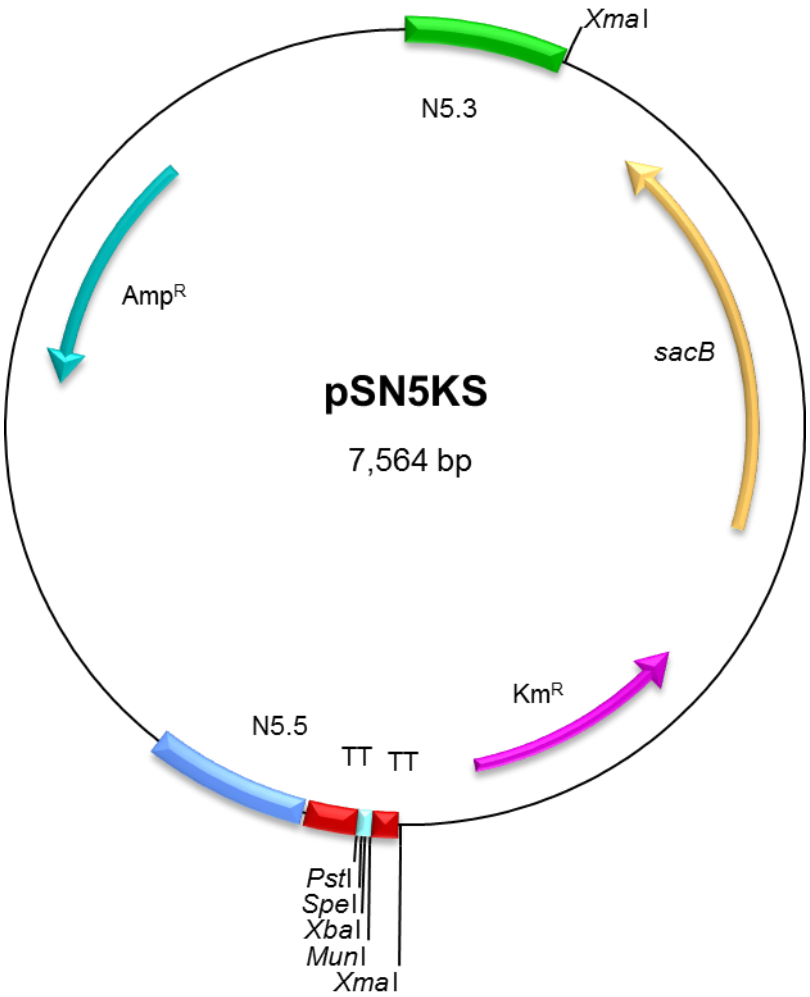
Supplementary Figure S11. Plasmid pSN16K. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N16.3 – 3' homologous region for site N16; N16.5 – 5' homologous region for site N16; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance.

A



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Supplementary Figure S12. Plasmid pSN5KS. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N5.3 – 3' homologous region for site N5; N5.5 – 5' homologous region for site N5; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; *sacB* – gene encoding the protein responsible for sucrose sensitivity.

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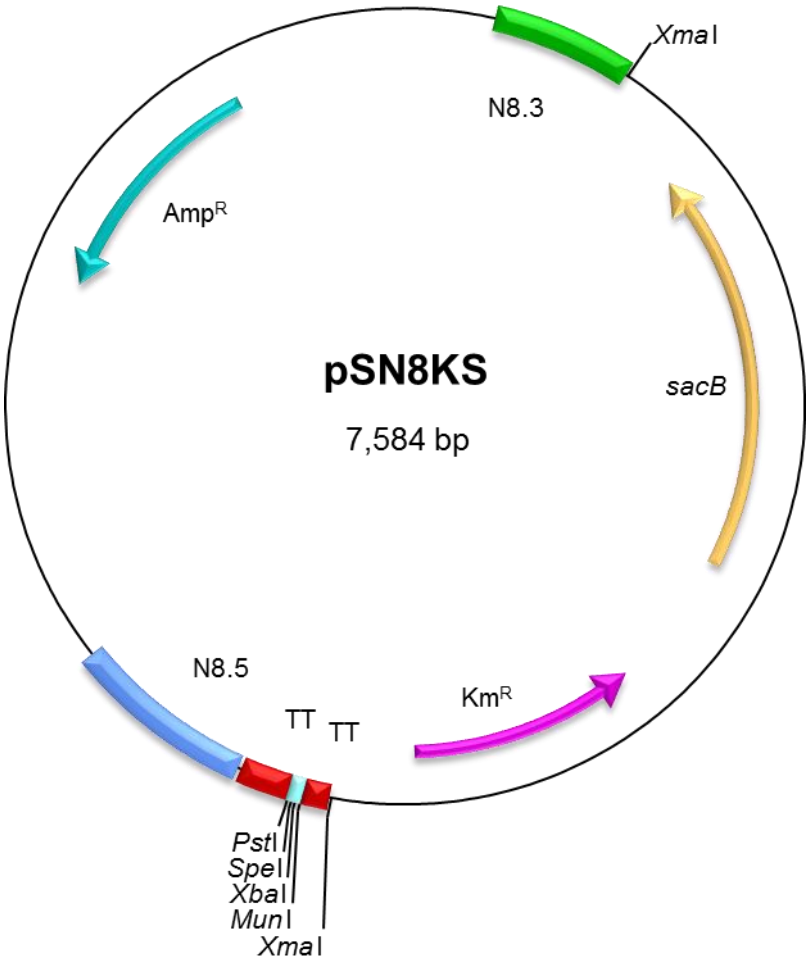

B

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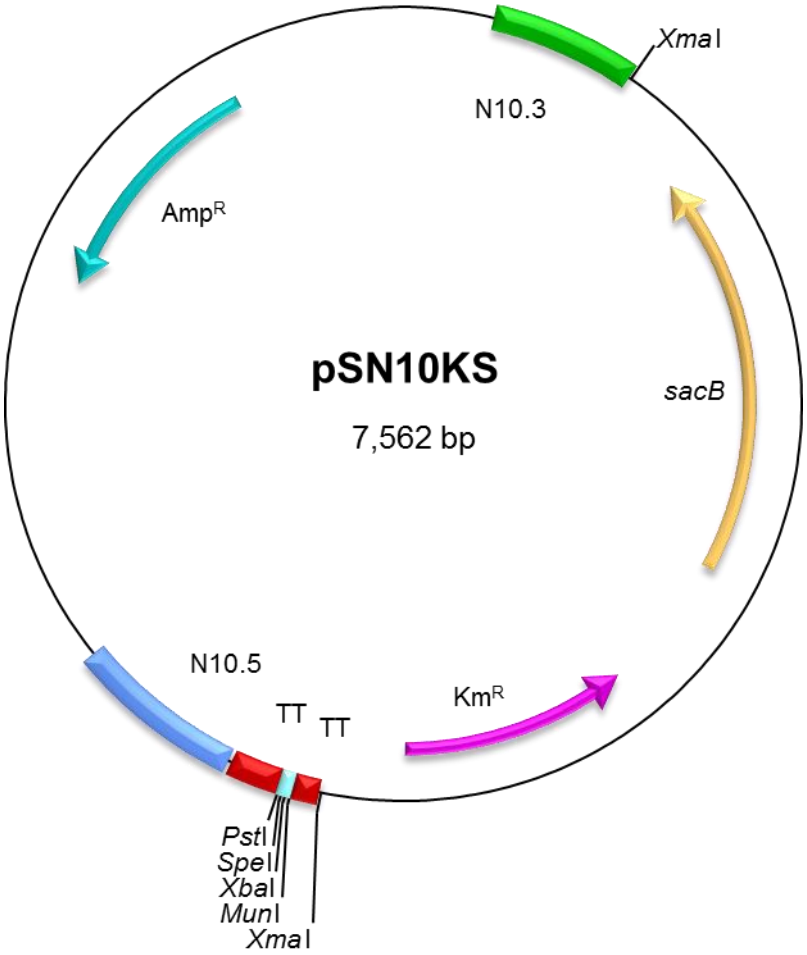
Supplementary Figure S13. Plasmid pSN8KS. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N8.3 – 3’ homologous region for site N8; N8.5 – 5’ homologous region for site N8; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; *sacB* – gene encoding the protein responsible for sucrose sensitivity.

A



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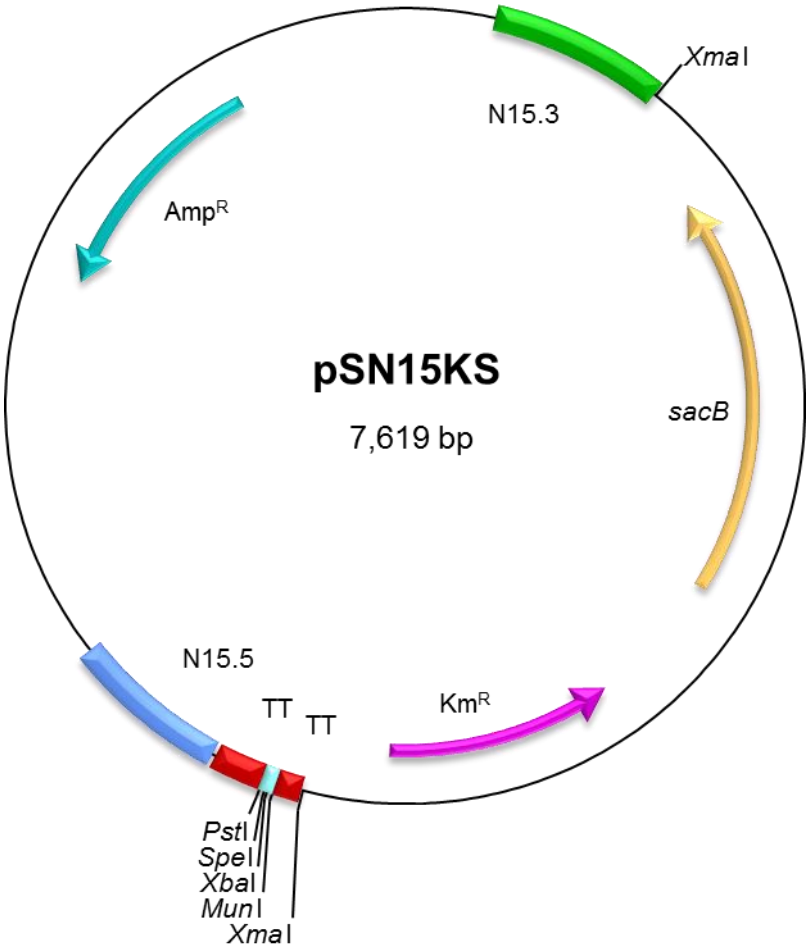
Supplementary Figure S14. Plasmid pSN10KS. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N10.3 – 3’ homologous region for site N10; N10.5 – 5’ homologous region for site N10; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; *sacB* – gene encoding the protein responsible for sucrose sensitivity.

A



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>pSN15KS

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Supplementary Figure S15. Plasmid pSN15KS. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N15.3 – 3’ homologous region for site N15; N15.5 – 5’ homologous region for site N15; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; *sacB* – gene encoding the protein responsible for sucrose sensitivity.

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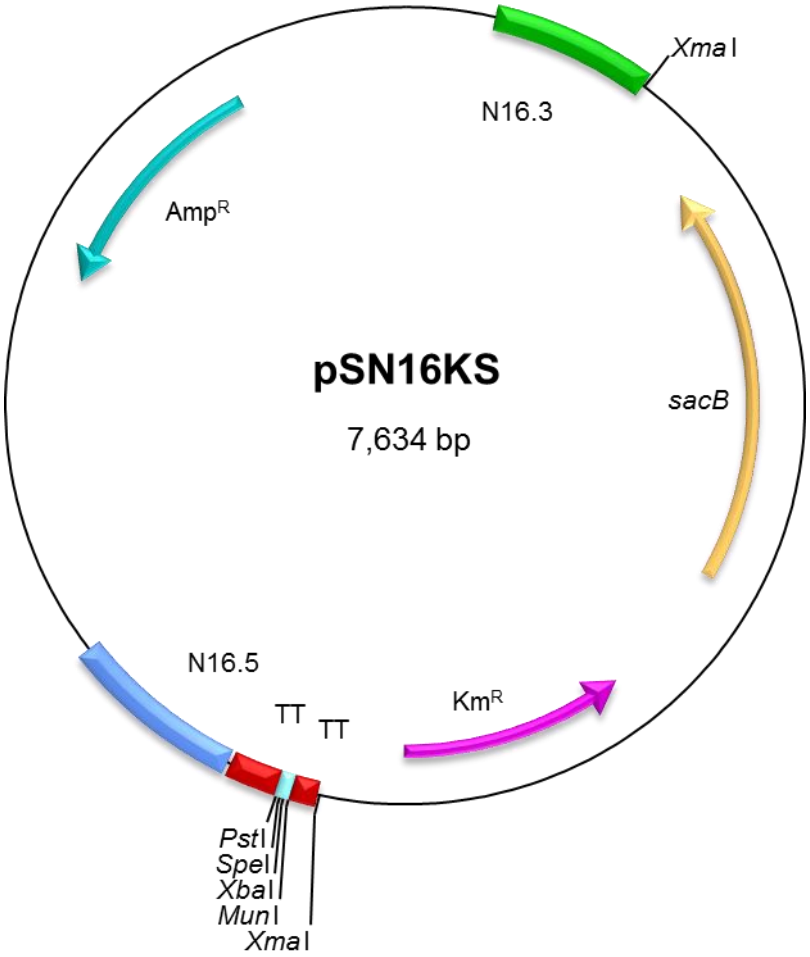

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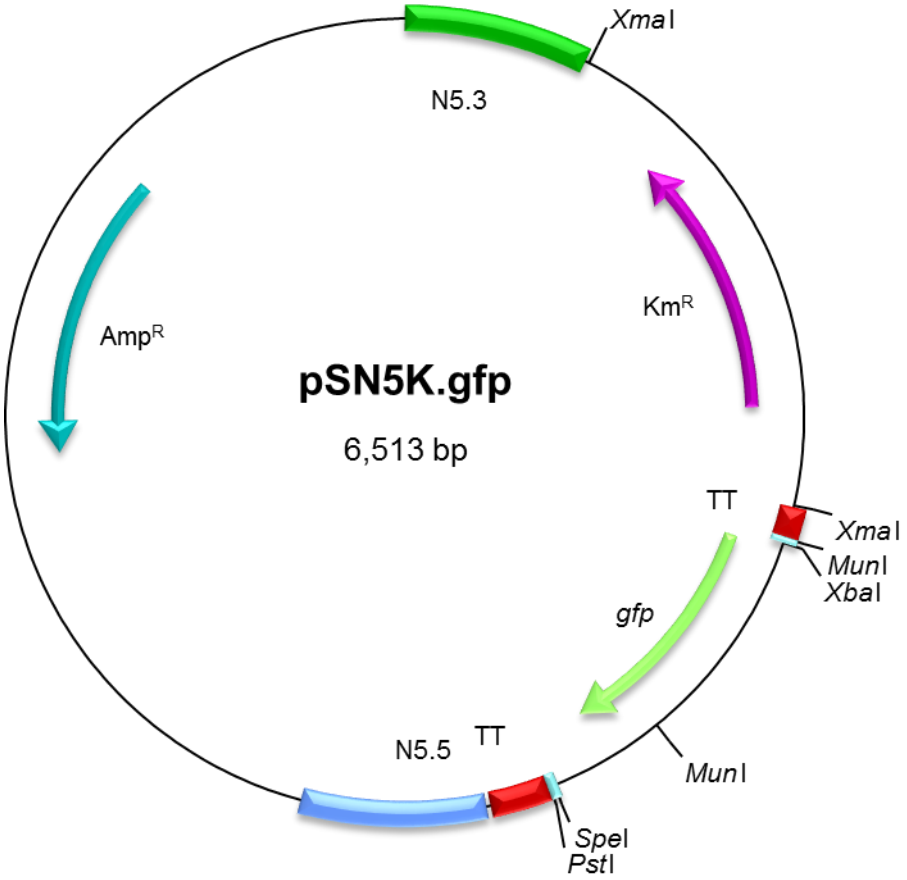
Supplementary Figure S16. Plasmid pSN16KS. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N16.3 – 3' homologous region for site N16; N16.5 – 5' homologous region for site N16; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; *sacB* – gene encoding the protein responsible for sucrose sensitivity.

A



B

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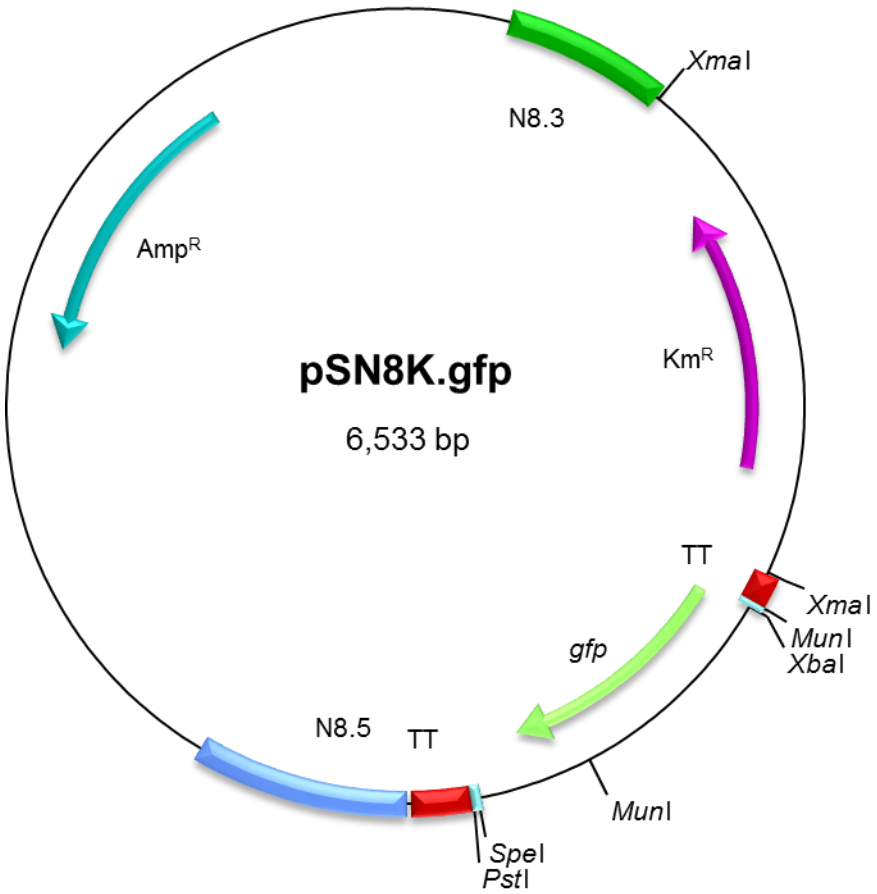
Supplementary Figure S17. Plasmid pSN5K.gfp. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N5.3 – 3' homologous region for site N5; N5.5 – 5' homologous region for site N5; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; *gfp* – sequence encoding the reporter GFP.

A



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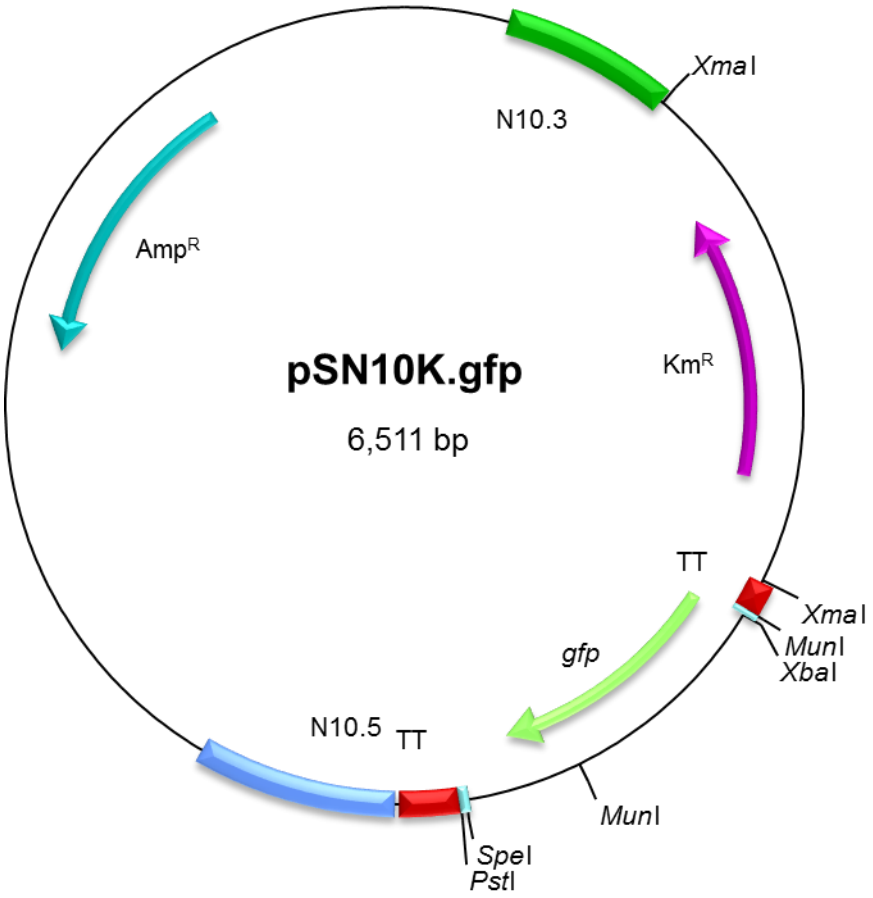
Supplementary Figure S18. Plasmid pSN8K.gfp. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N8.3 – 3' homologous region for site N8; N8.5 – 5' homologous region for site N8; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; *gfp* – sequence encoding the reporter GFP.

A



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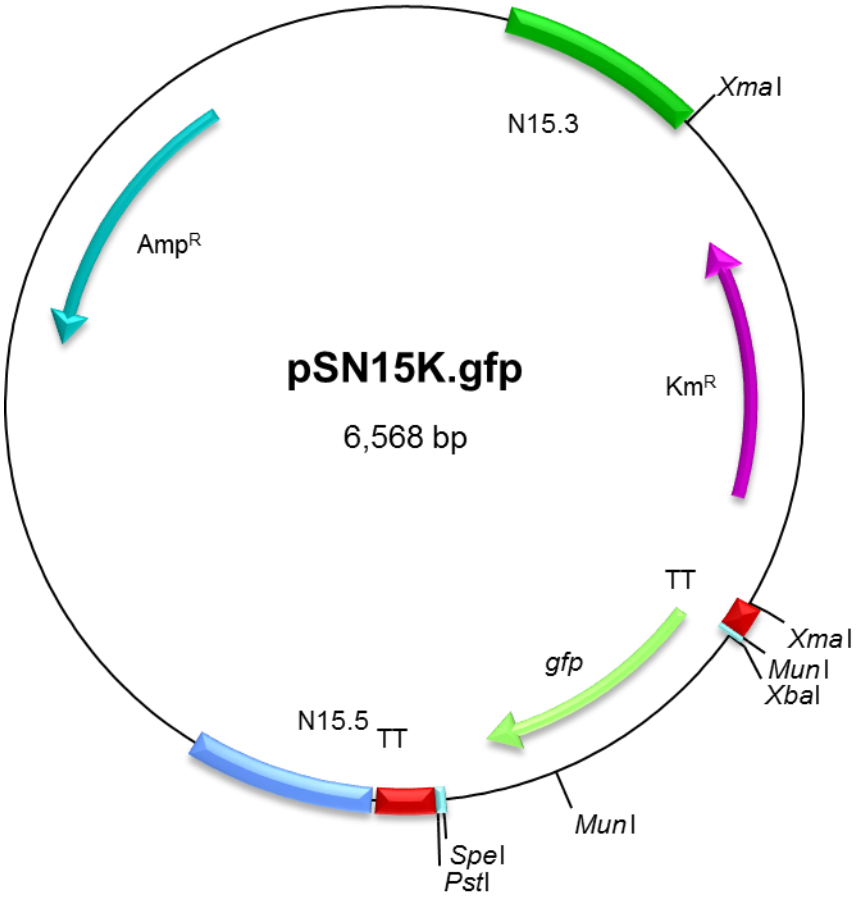
Supplementary Figure S19. Plasmid pSN10K.gfp. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N10.3 – 3' homologous region for site N10; N10.5 – 5' homologous region for site N10; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; *gfp* – sequence encoding the reporter GFP.

A



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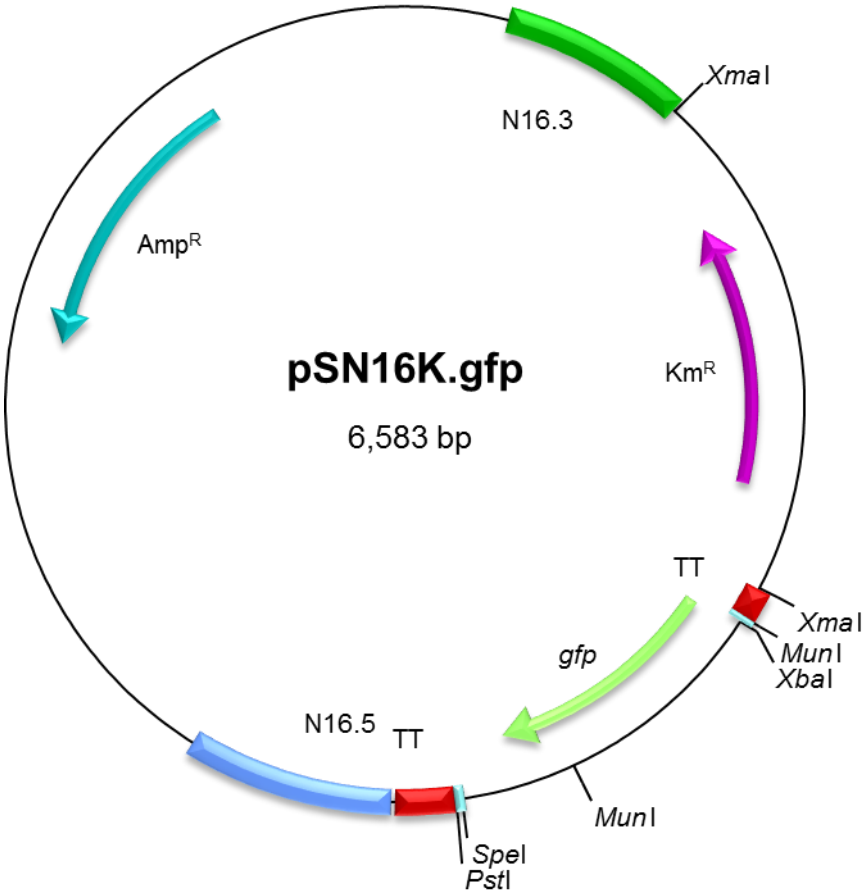
Supplementary Figure S20. Plasmid pSN15K.gfp. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N15.3 – 3' homologous region for site N15; N15.5 – 5' homologous region for site N15; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; *gfp* – sequence encoding the reporter GFP.

A



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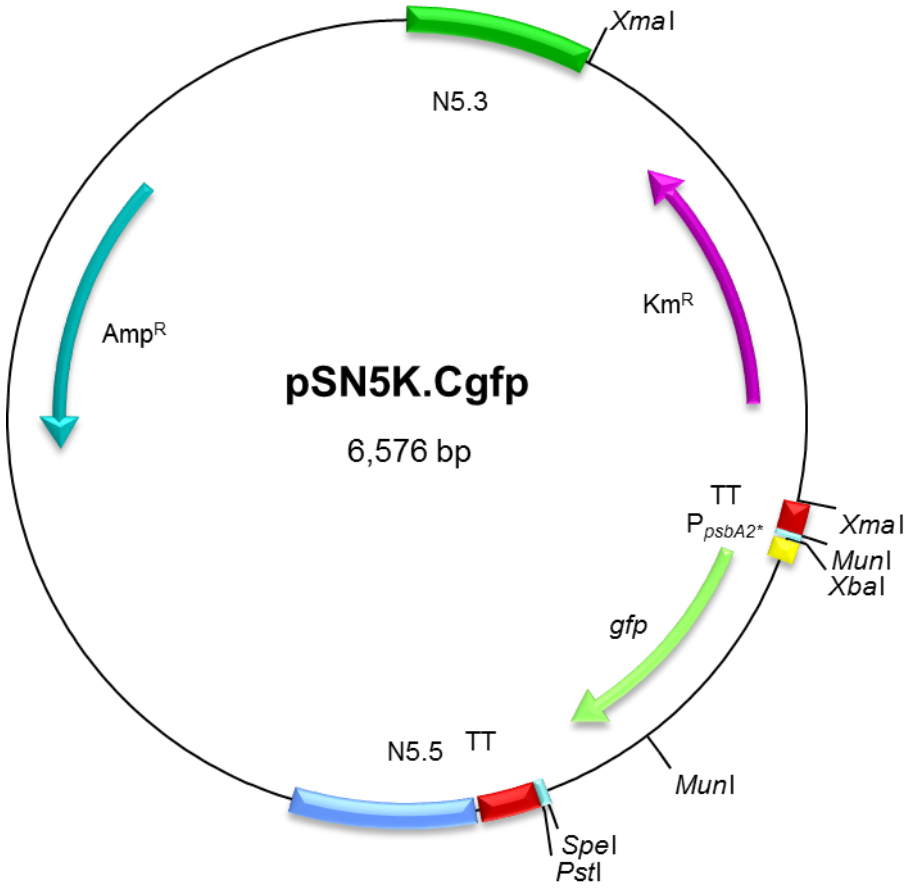
Supplementary Figure S21. Plasmid pSN16K.gfp. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N16.3 – 3' homologous region for site N16; N16.5 – 5' homologous region for site N16; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; *gfp* – sequence encoding the reporter GFP.

A



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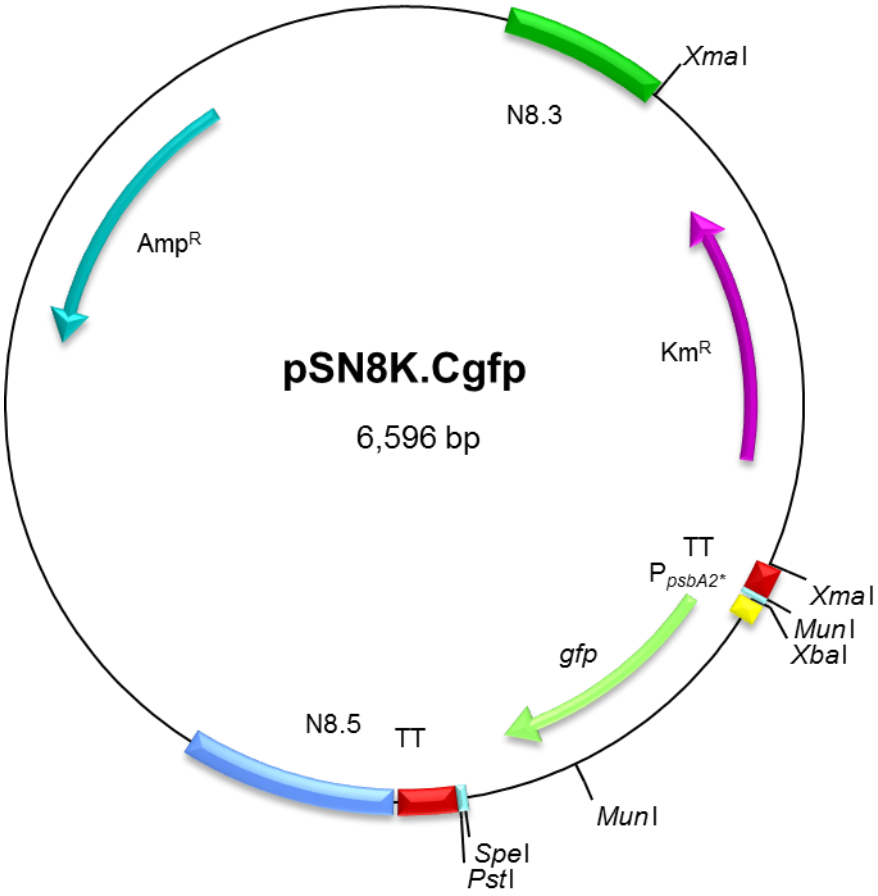
Supplementary Figure S22. Plasmid pSN5K.Cgfp. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N5.3 – 3' homologous region for site N5; N5.5 – 5' homologous region for site N5; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; P_{psba2*} – synthetic minimal constitutive promoter based on the native promoter of *psba2* gene; *gfp* – sequence encoding the reporter GFP.

A



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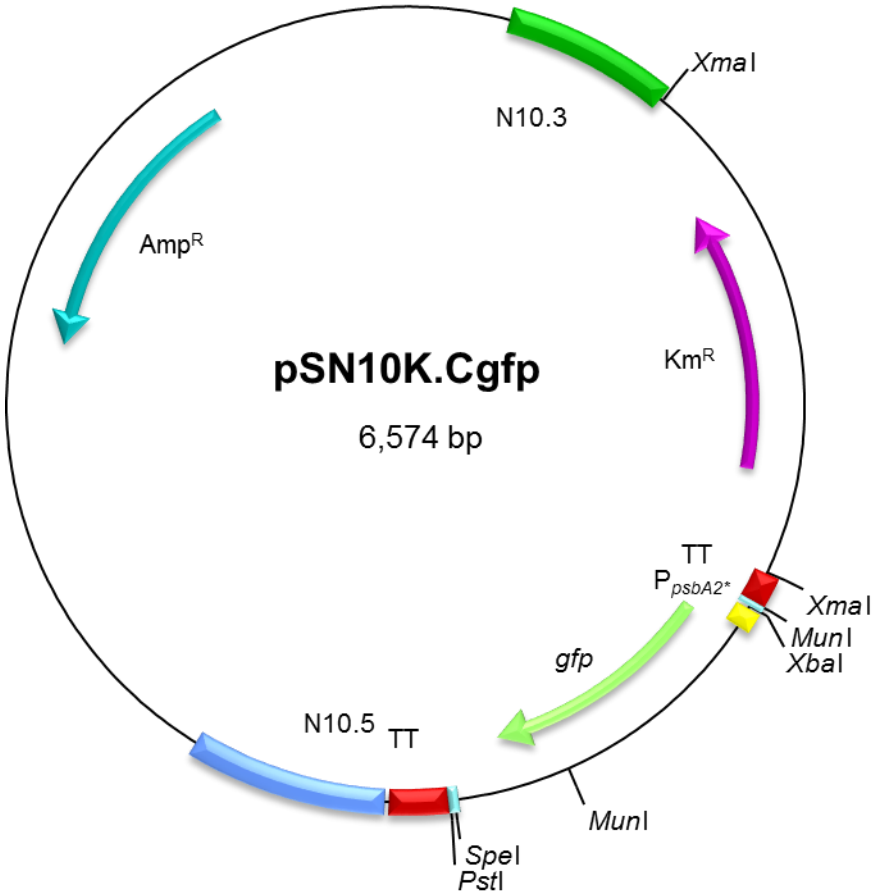
Supplementary Figure S23. Plasmid pSN8K.Cgfp. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N8.3 – 3' homologous region for site N8; N8.5 – 5' homologous region for site N8; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; P_{psba2*} – synthetic minimal constitutive promoter based on the native promoter of *psba2* gene; *gfp* – sequence encoding the reporter GFP.

A



B

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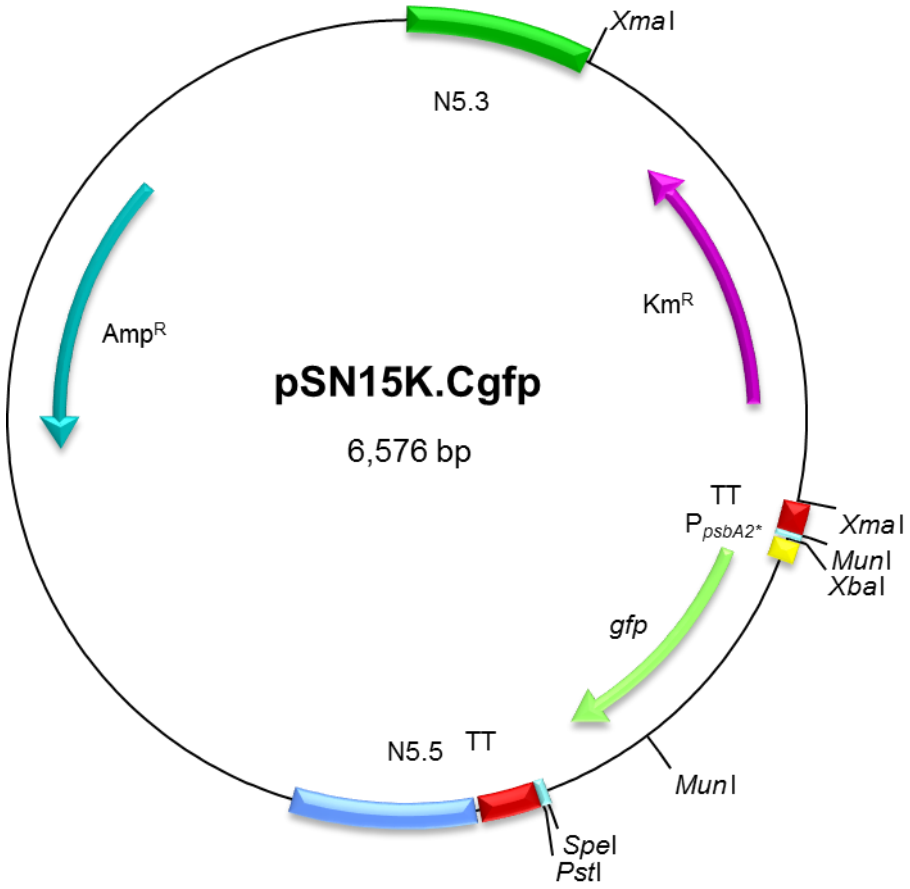
Supplementary Figure S24. Plasmid pSN10K.Cgfp. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N10.3 – 3' homologous region for site N10; N10.5 – 5' homologous region for site N10; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; P_{psba2*} – synthetic minimal constitutive promoter based on the native promoter of *psba2* gene; *gfp* – sequence encoding the reporter GFP.

A



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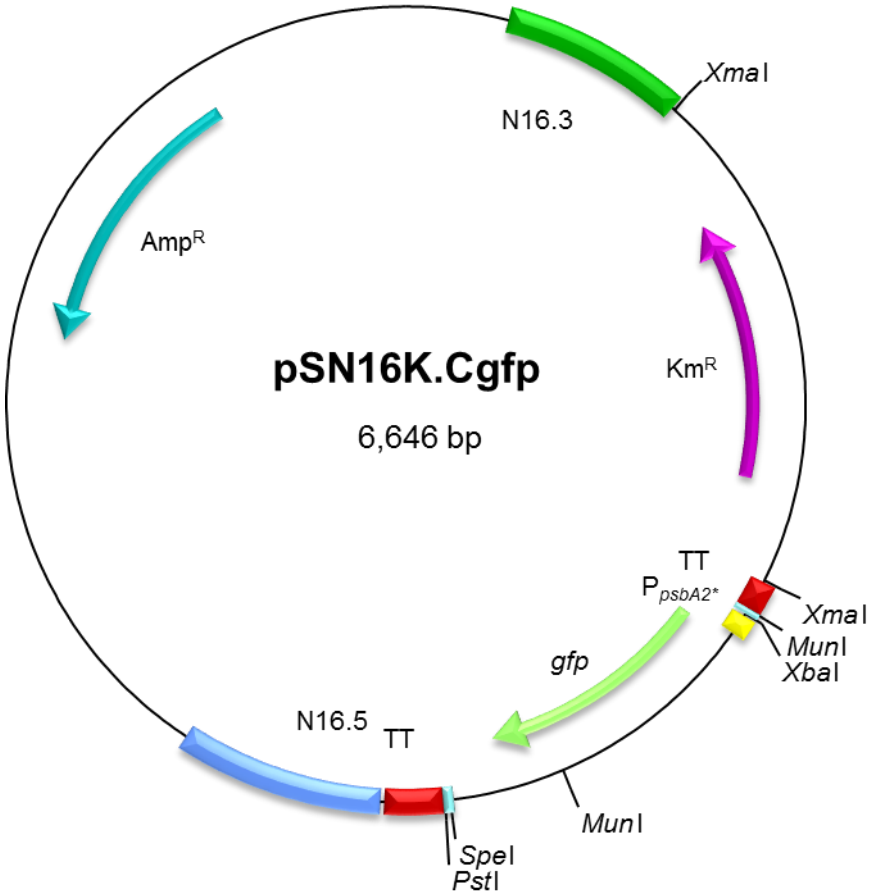
Supplementary Figure S25. Plasmid pSN15K.Cgfp. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N15.3 – 3' homologous region for site N15; N15.5 – 5' homologous region for site N15; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; P_{psba2*} – synthetic minimal constitutive promoter based on the native promoter of *psba2* gene; *gfp* – sequence encoding the reporter GFP.

A



B

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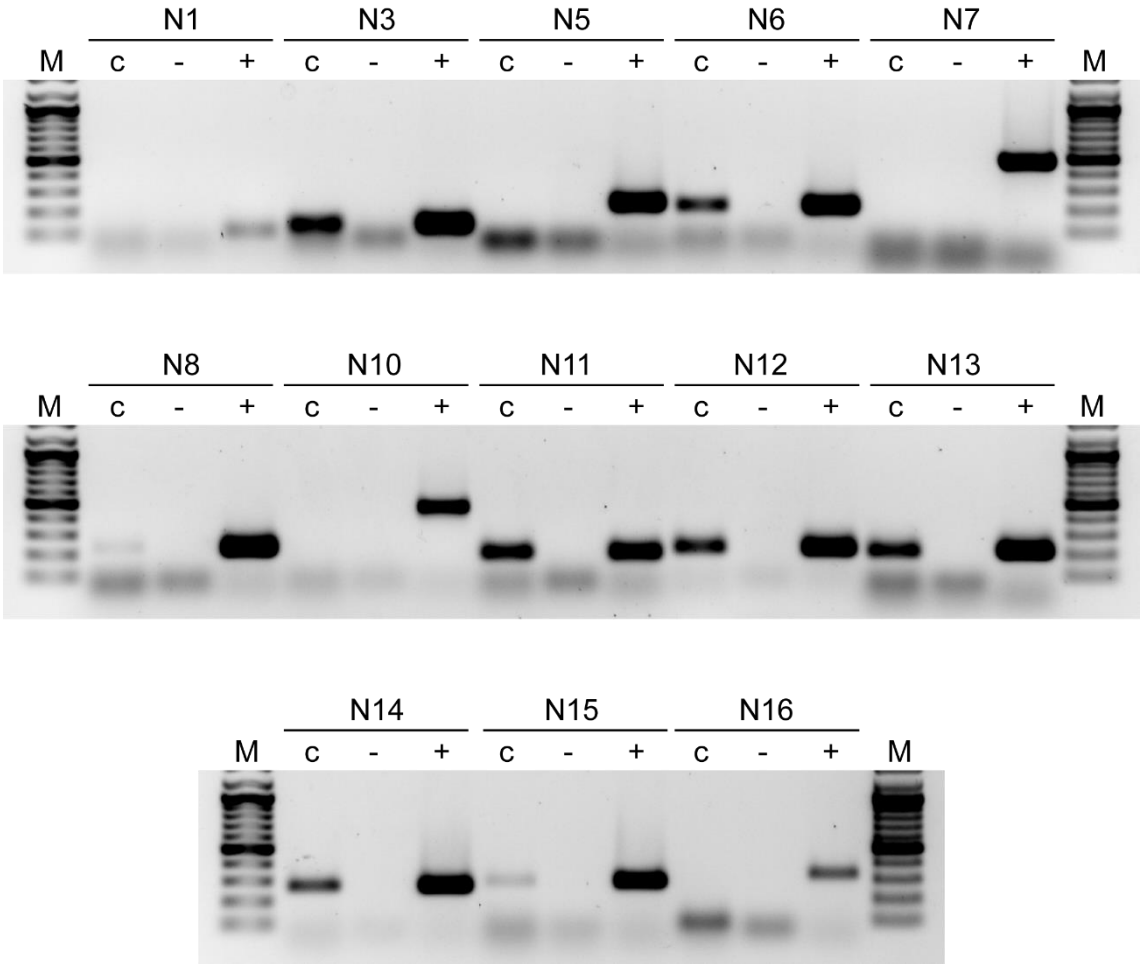
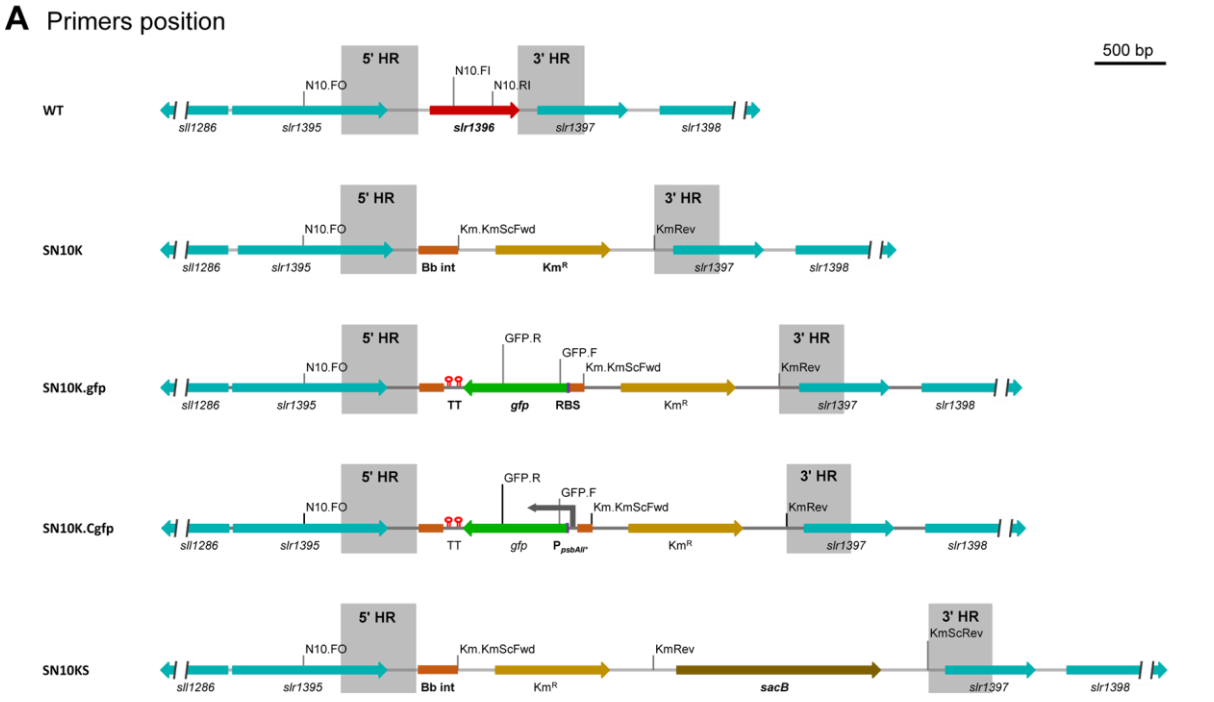
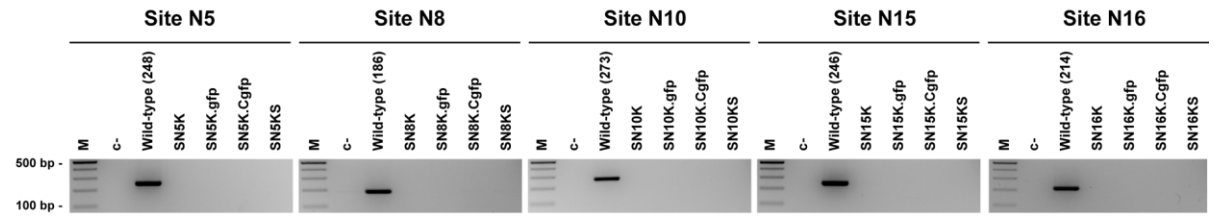
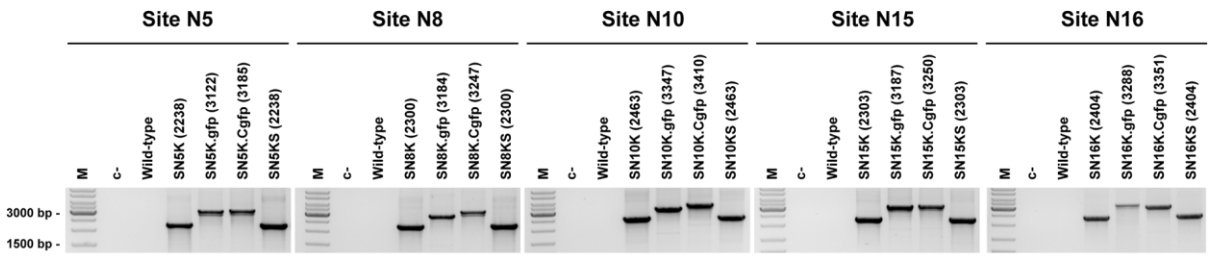
Supplementary Figure S26. Plasmid pSN16K.Cgfp. The plasmid map with the main features highlighted is depicted in **A** and the FASTA formatted sequence in **B**. N16.3 – 3' homologous region for site N16; N16.5 – 5' homologous region for site N16; TT – double transcriptional terminators; Amp^R – gene encoding the protein responsible for ampicillin resistance; Km^R – gene encoding the protein responsible for neomycin/kanamycin resistance; P_{psba2*} – synthetic minimal constitutive promoter based on the native promoter of *psba2* gene; *gfp* – sequence encoding the reporter GFP.

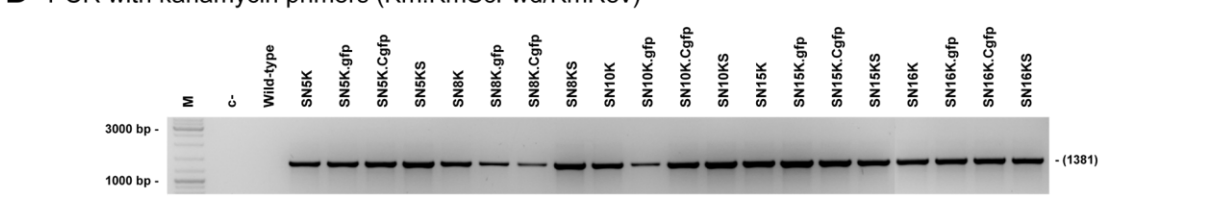


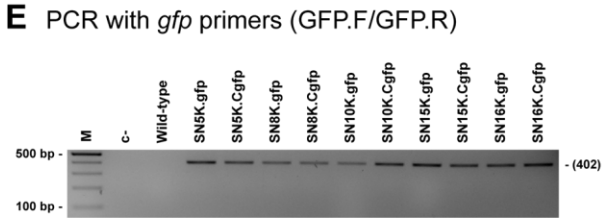
Supplementary Figure S27. RT-PCR transcription analysis of thirteen loci (N1, N3, N5, N6, N7, N8, N10, N11, N12, N13, N14, N15, N16). *Synechocystis* wild-type samples for RNA extraction were collected at OD_{730}≈0.8–0.9. These results are representative of the three biological triplicates and technical duplicates. c - cDNA, - negative control (absence of template), + positive control (genomic DNA), M – GeneRuler DNA ladder (Thermo Fisher Scientific).

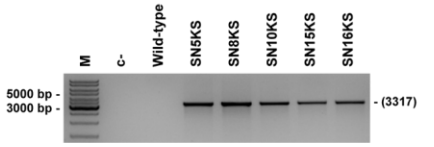

A Primers position
500 bp
WT
SN10K
SN10K.gfp
SN10K.Cgfp
SN10KS
5' HR
3' HR
N10.FO
N10.FI
N10.RI
Km.KmScFwd
KmRev
KmScRev
GFP.R
GFP.F
sll1286
slr1395
slr1396
slr1397
slr1398
Bb int
TT
gfp
RBS
sacB


B PCR with inner primers (Nn.FI/Nn.RI)
Site N5
Site N8
Site N10
Site N15
Site N16
M
c-
Wild-type (248)
SN5K
SN5K.gfp
SN5K.Cgfp
SN5KS
Wild-type (186)
SN8K
SN8K.gfp
SN8K.Cgfp
SN8KS
Wild-type (273)
SN10K
SN10K.gfp
SN10K.Cgfp
SN10KS
Wild-type (246)
SN15K
SN15K.gfp
SN15K.Cgfp
SN15KS
Wild-type (214)
SN16K
SN16K.gfp
SN16K.Cgfp
SN16KS
500 bp -
100 bp -

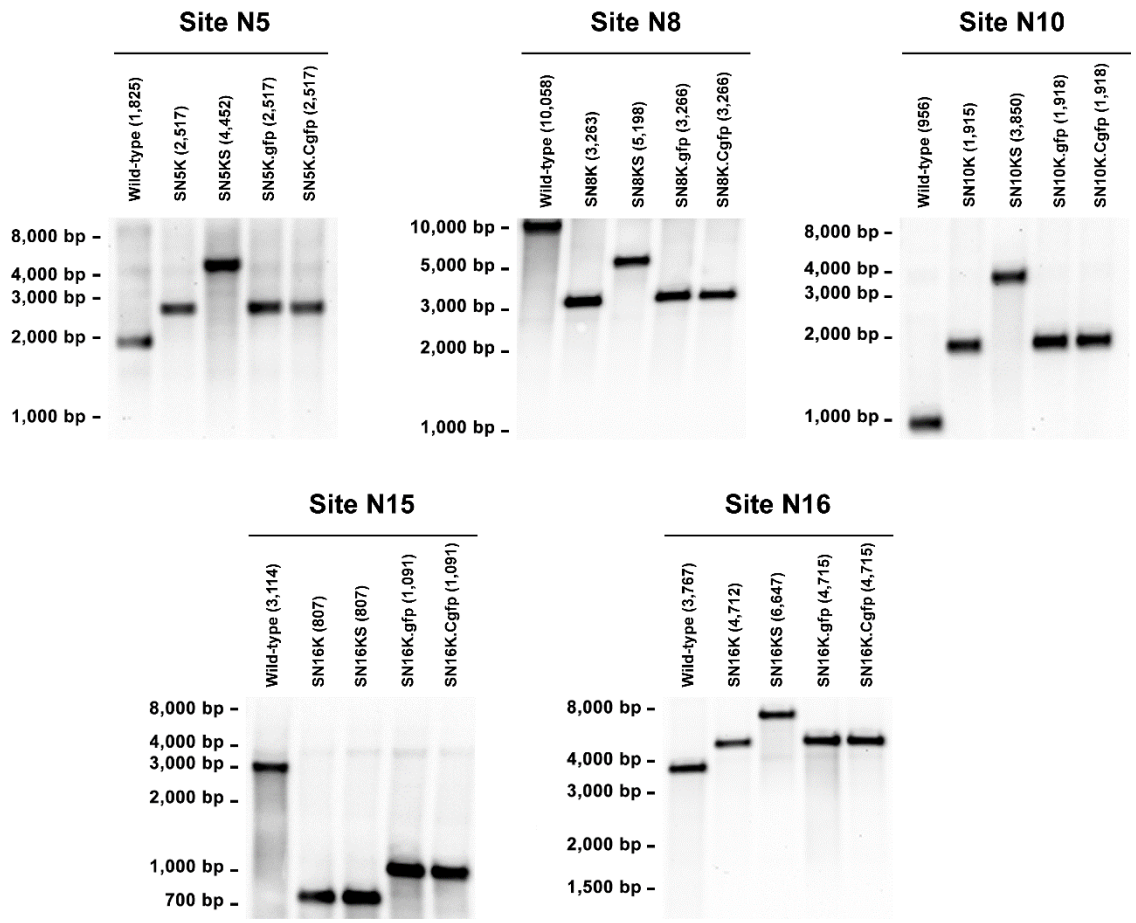

C PCR with outer and internal primers (Nn.FO/KmRev)
Site N5
Site N8
Site N10
Site N15
Site N16
M
c-
Wild-type
SN5K (2238)
SN5K.gfp (3122)
SN5K.Cgfp (3185)
SN5KS (2238)
SN8K (2300)
SN8K.gfp (3184)
SN8K.Cgfp (3247)
SN8KS (2300)
SN10K (2463)
SN10K.gfp (3347)
SN10K.Cgfp (3410)
SN10KS (2463)
SN15K (2303)
SN15K.gfp (3187)
SN15K.Cgfp (3250)
SN15KS (2303)
SN16K (2404)
SN16K.gfp (3288)
SN16K.Cgfp (3351)
SN16KS (2404)
3000 bp -
1500 bp -


D PCR with kanamycin primers (Km.KmScFwd/KmRev)
M
c-
Wild-type
SN5K
SN5K.gfp
SN5K.Cgfp
SN5KS
SN8K
SN8K.gfp
SN8K.Cgfp
SN8KS
SN10K
SN10K.gfp
SN10K.Cgfp
SN10KS
SN15K
SN15K.gfp
SN15K.Cgfp
SN15KS
SN16K
SN16K.gfp
SN16K.Cgfp
SN16KS
3000 bp -
1000 bp -
- (1381)

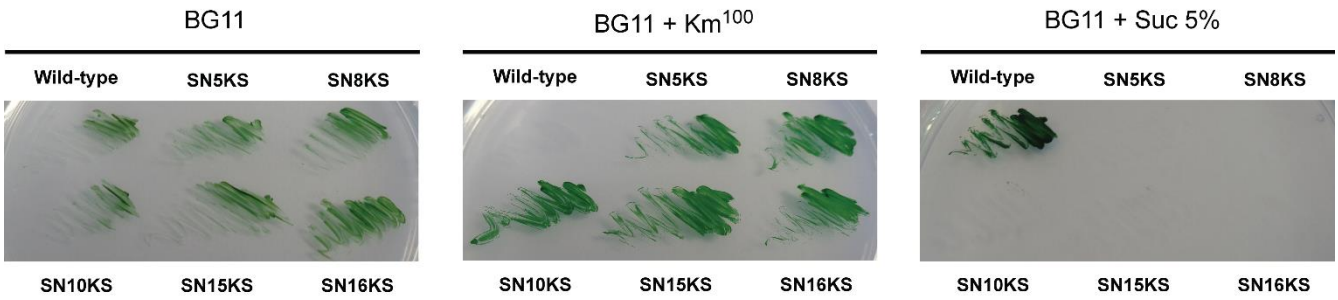

E PCR with gfp primers (GFP.F/GFP.R)
M
c-
Wild-type
SN5K.gfp
SN5K.Cgfp
SN8K.gfp
SN8K.Cgfp
SN10K.gfp
SN10K.Cgfp
SN15K.gfp
SN15K.Cgfp
SN16K.gfp
SN16K.Cgfp
500 bp -
100 bp -
- (402)


F PCR with kanamycin/sacB primers (Km.KmScFwd/KmScRev)
M
c-
Wild-type
SN5KS
SN8KS
SN10KS
SN15KS
SN16KS
5000 bp -
3000 bp -
- (3317)

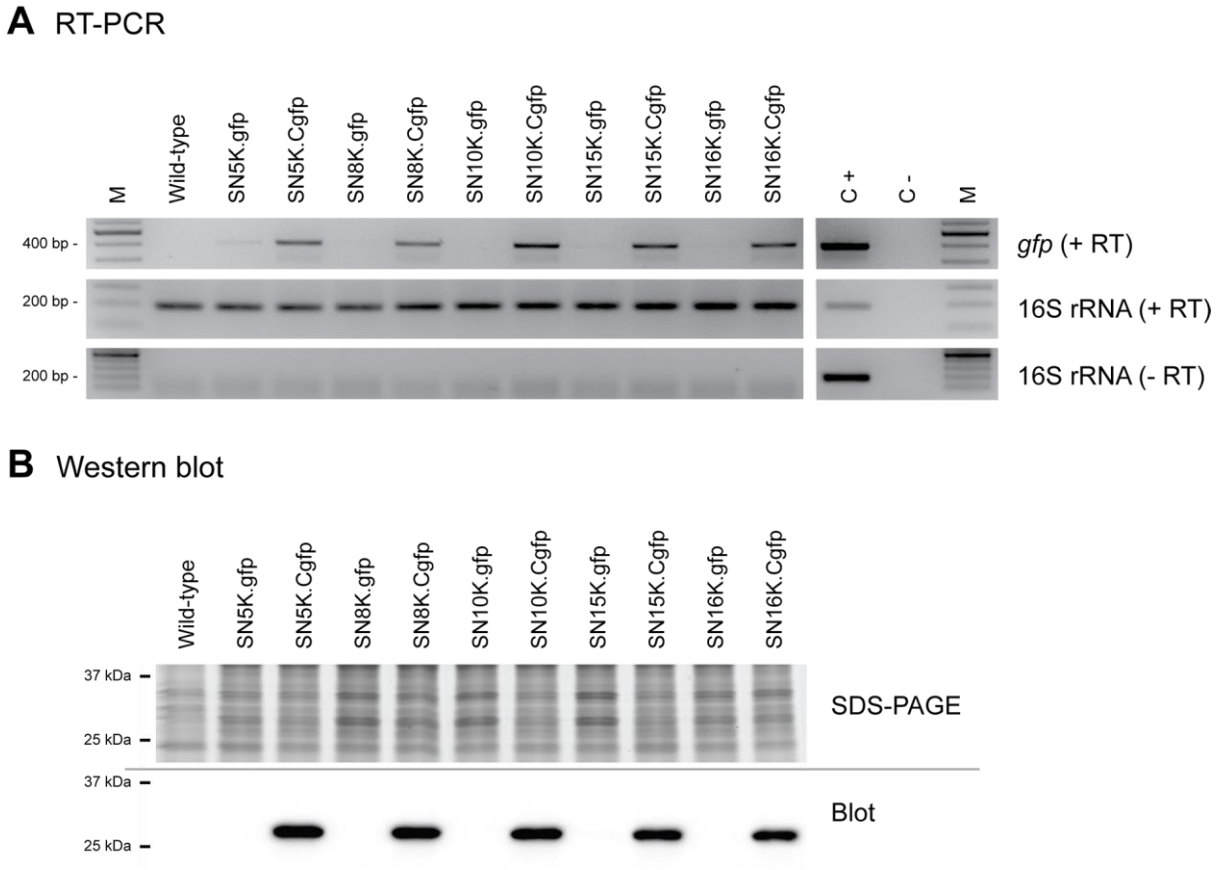
Supplementary Figure S28. PCR confirmation of the full segregation of the *Synechocystis* mutants in the five neutral sites (N5, N8, N10, N15, N16). Schematic representation of the position of the primers used for the wild-type and mutants, exemplified for N10 (**A**). PCR reactions were performed using primers within the ORF corresponding to each neutral site (**B**), a primer external to each site and a primer within the selection cassette (**C**), primer amplifying the kanamycin resistance cassette (**D**), primers amplifying the within the *gfp* gene (**E**) or primers amplifying the double selection cassette (**F**) (listed in **Supplementary Table 6**). No template controls were always included (c-) and the expected band sizes are indicated. M – GeneRuler DNA ladder (Thermo Fisher Scientific).



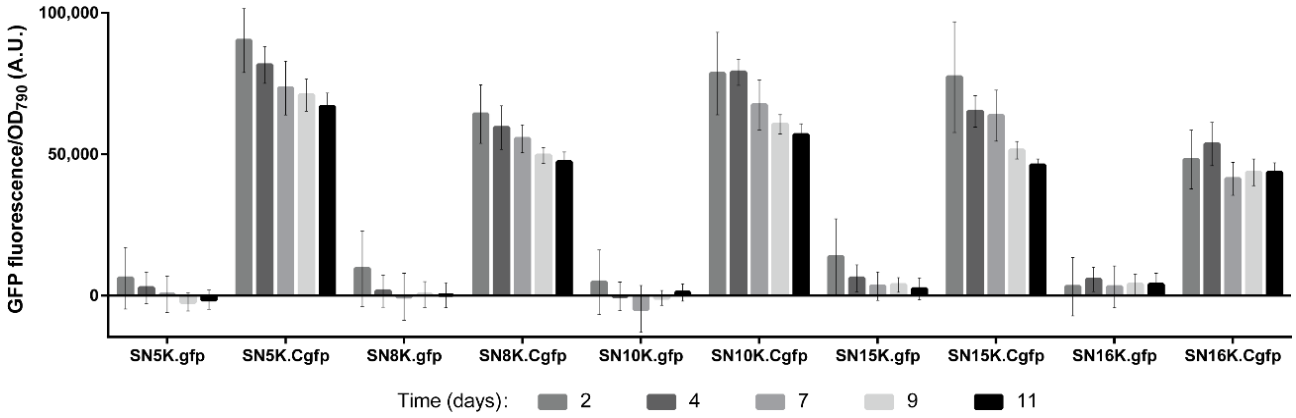
Supplementary Figure S29. Southern blot confirmation of the full segregation of the *Synechocystis* mutants in the five neutral sites (N5, N8, N10, N15, N16). Expected band sizes are within brackets. For details see Material and Methods.



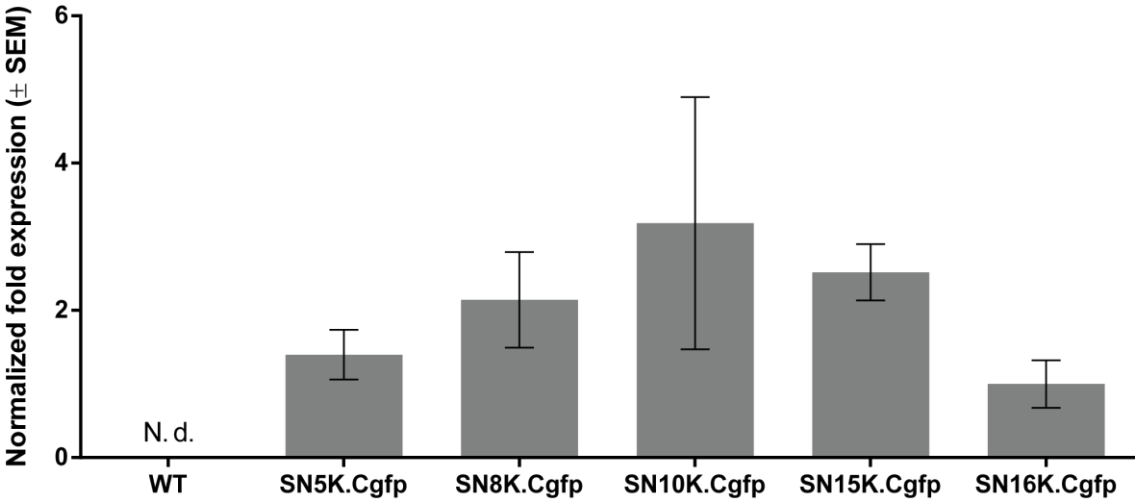
Supplementary Figure S30. Phenotypic characterization of mutants harboring the double selection cassette conferring kanamycin resistance and sucrose sensitivity (SN*n*KS). *Synechocystis* wild-type and mutants were grown in agar plates containing BG11 medium (BG11), BG11 medium supplemented with 100 µg mL^{-1} kanamycin (BG11 + Km^{100}) and BG11 medium supplemented with 5% (wt/vol) sucrose (BG11 + Suc 5%).



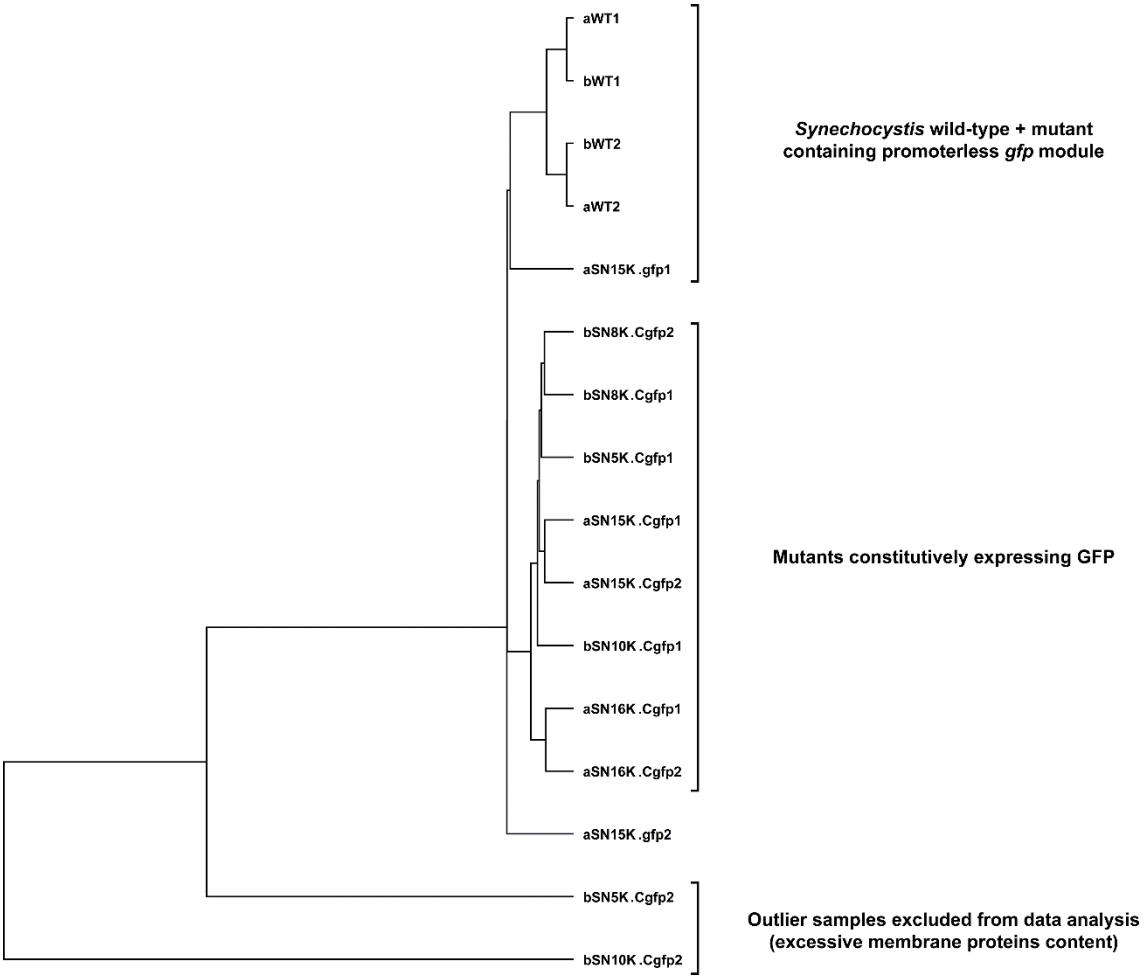
Supplementary Figure S31. Detection of *gfp* transcripts and GFP in *Synechocystis* mutants harboring a promoterless *gfp* synthetic module (SN*n*K.gfp) or a module with *gfp* under the control of the synthetic constitutive promoter P_{psba2*} (SN*n*K.Cgfp). *gfp* transcription was assessed by RT-PCR (**A**) and GFP expression was assessed by Western blot (**B**). Wild-type was included as control. M – GeneRuler DNA ladder (Thermo Fisher Scientific); C + – Positive control (wild-type gDNA for 16S rRNA and pSB1A2-E0240 for *gfp*); C - – Negative control (no template); - RT – PCRs performed using RNA as template; + RT – PCRs performed using cDNA as template. For details see Online Methods.



Supplementary Figure S32. Detection of GFP expression in *Synechocystis* mutants harboring a promoterless *gfp* synthetic module (SNnK.gfp) or a module with *gfp* under the control of a synthetic constitutive promoter (SNnK.Cgfp). Total cell fluorescence was measured 2, 4, 7, 9 and 11 days after inoculation, using 200 µL of three independent culture replicates. Measurements were performed in triplicate and fluorescence was normalized to OD_{790}. Normalized fluorescence from the wild-type was used as baseline. Bars indicate mean ± s.d.



Supplementary Figure S33. Normalized fold expression of *gfp* (RT-qPCR) in *Synechocystis* mutants harboring a synthetic module with *gfp* under the control of a synthetic constitutive promoter. WT – wild-type (control). Data from 3 biological replicates and 3 technical replicates were normalized against three reference genes (16S, *petB*, and *rnpB*) and error bars represent ± s.e.m.



Supplementary Figure S34. Dendrogram plot showing the hierarchical clustering of the iTRAQ labels following the combination of the data from the two experiments. All samples beginning with “a” are from the first iTRAQ, whilst those beginning with “b” are from the second.