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         | CATGGATTGGAAGGAGGG | 100                |
| N1R         | TTTGGTGAACATTAATGAC |                   |
| N3F         | GCACTTTACTCCTGAAAC | 129                |
| N3R         | AAGCTGATTTCTAGTAAGTG |                   |
| N5F         | GGAAGGTTTGTGGCTGCT | 232                |
| N5R         | GTCATTTGAGCAGATTG |                    |
| N6F         | AAGGTTAATCAACGAGAAATG |            |
| N6R         | ATTTGTAACGACCTTCC |                    |
| N7F         | TAATGTGAGAATCTTACCC |                  |
| N7R         | ACTAACGATGGAATCTTG | 500                |
| N8F         | AACTTAACTCCTATCGTAG |                  |
| N8R         | GAAACGTTGATAAGCAGTC | 231                |
| N10F        | GATATTGATGTGATTGC | 478                |
| N10R        | TTTATCTCTGTCTCC |                    |
| N11F        | TGAATACCACATCTCCATTGC |               |
| N11R        | TTTACGCTGGCTGTGGAC | 200                |
| N12F        | TTCTACACCGCTACGTGTC |                  |
| N12R        | CACTTTATCAGCAGGAC | 227                |
| N13F        | CCCATGATGACCAAGAG | 216                |
| N13R        | CTAGTAACTCGTGTTCCTC |               |
| N14F        | AAGTGTTGCAAGGCAATGG | 270                |
| N14R        | TCACCAATGCGATTTTCC |                   |
| N15F        | TTAATTGACCGCTTCCTAC |             |
| N15R        | TAGAGACACGCGATGTC | 293                |
| N16F        | TGATGTGAGGCAAGGATG | 335                |
| N16R        | GGGCTAAGGGAATATGG |                    |
### Supplementary Table S2. Other primers used in this work

| Primer name | Sequence 5' → 3'' | Purpose |
|-------------|-------------------|---------|
| Km.KmScFwd  | CTGACCCCGGTTGTAATGTCAGCTACTGG | Selection cassettes amplification |
| KmRev       | CAAACCGCCGGATTTACTTTTGGACCTC | |
| KmScRev     | ACAGACCCCGGAAACGGGATGGCTGATG | |
| N5.5O       | GGGGCCCTCGAGTGGGGGCACTGCTAGGCAATTTTG | |
| N5.5I       | GATTACAACCCGGGTGTAATCCGGTTTGTATTTTACGTGTCGCCCCTGGG | |
| N5.3O       | GTTCCAAATCGAGTCTGCTGGAGACTGC | |
| N5.3I       | GATTACAACCCGGGTGTAATCCCTTATCCTGGGCTAATCGGATTTAGGGATTTTG | |
| N8.5O       | GATGACCGCTGGCGGAGTTAGTCCAG | |
| N8.5I       | GATTACAACCCGGGTGTAATCCCTTGCTGCTTTCATTCCCCTATTGCCGAAGCTGCTTAGAGAATGT | |
| N10.5O      | GATAACCTCGAGTCCCGGGTGAATTTATGCAACATCG | |
| N10.5I      | GATTACAACCCGGGTGTAATCCAACTCAGCCTTCTAAATGCAGGAATG | |
| N10.3O      | GATGACCGCTGGCGGAGTTAGTCCAG | |
| N10.3I      | GATTACAACCCGGGTGTAATCCCTTATCCTGGGCTAATCGGATTTAGGGATTTTG | |
| N5.FO       | TTCCTGGTAACTCAGCTAC | |
| N5.FI       | AGCCAGTTCAAGGGAGATGTTTGTTG | |
| N5.RI       | CCGGTGTCGCTGGACGGTACTGGTG | |
| N8.FO       | CCCAGTTAAAACGCTGGGAAGAG | |
| N8.FI       | TCCGCAAGCTTTTCAGAAC | Mutants confirmation by PCR |
| N8.RI       | CAAACTCAGCGGGGAAAC | |
| N10.FO      | CCGGTGTCGCTTTTACGGGAAACGGATG | |
| N10.FI      | GCATGCGTACCTGGTTGAC | |
| N10.RI      | CCGGTGTCGCTTTTACGGGAAACGGATG | |
| N15.FO      | CTCCAAGGCCGACTACCTTC | Site directed mutagenesis |
| N15.FI      | CCCAGTGGAATGCGATCAC | |

**Note:** The primers are designed for specific purposes, including selection cassettes amplification, amplification of the DNA fragments flanking the neutral sites, Site directed mutagenesis, and Mutants confirmation by PCR.
| Primer ID | Sequence                                      | Function                              |
|----------|-----------------------------------------------|---------------------------------------|
| N15.RI   | TAGGAGGGCGATCCCGAAG                            | Mutants confirmation by PCR           |
| N16.FO   | ACCCATTTCTGGGAGTAGG                           |                                      |
| N16.FI   | GCCTTGGTGCCCCTGACGTAGGTG                      |                                      |
| N16.RI   | GACCGATCGCCGCAGTGTCTG                        |                                      |
| GFP.F    | TCTTGTTGAATTAGATGG                            | RT-PCR/RT-qPCR                       |
| GFP.R    | TGTGATTATAGGTGATTCC                          |                                      |

*Restriction sites underlined
*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 | 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        | ssr0060| 2684766 - 2684990   | d           | 74                                                     |
| N5        | ssl1476| 3398409 - 3398717   | c           | 102                                                    |
| N6        | slr1669| 1212609 - 1213358   | d           | 249                                                    |
| N7        | ssl0494| 3224429 - 3225334   | c           | 301                                                    |
| N8        | slr0573| 2816517 - 2816960   | d           | 147                                                    |
| N9        | ssl0181| 2737929 - 2738552   | c           | 207                                                    |
| N10       | slr1396| 707437 - 708054     | d           | 205                                                    |
| N11       | ssr1038| 2950107 - 2950343   | d           | 78                                                     |
| N12       | ssl3615| 776058 - 776342     | c           | 94                                                     |
| N13       | slr0587| 3574056 - 3576422   | d           | 110                                                    |
| N14       | ssl0167| 2317536 - 2318030   | c           | 164                                                    |
| N15       | slr0271| 1524568 - 1525086   | d           | 172                                                    |
| N16       | slr0397| 2146801 - 2147376   | d           | 191                                                    |

*d – direct sequence, c – complement sequence.
Supplementary Table S4. ANOVA analysis of *Synechocystis* wild-type growth compared to the SNnK mutants. *P*-values of up to second order interactions are shown.

| 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*                | 4.58x10^{-15} | 1.24x10^{-04} | 8.65x10^{-03} | 3.37x10^{-01} | 3.48x10^{-09} |
| Light + Glucose          | 2.57x10^{-10} | 1.36x10^{-14} | 3.62x10^{-18} | 6.46x10^{-20} | 9.58x10^{-16} |
| Light + Mutation         | 1.74x10^{-23} | 4.51x10^{-04} | 1.04x10^{-01} | 5.27x10^{-01} | 7.76x10^{-02} |
| Glucose + Mutation       | 1.74x10^{-37} | 1.00x10^{-01} | 1.20x10^{-01} | 1.44x10^{-04} | 1.30x10^{-61} |

*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<sup>a</sup> |               |
| 114   | WT2<sup>a</sup> |               |
| 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   |

<sup>a</sup>Samples common to both iTRAQ experiments
| 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. |
| 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}). |
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 Xhol and SalI 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 Xmal 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 Xmal 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.
ACGTTCTTCGCGGAGAAAACCTCTCAAGGATCTTACCGGTAACCCCACTGATTTACCATCTTTTTTCCCAATCTTTGAAAGCCTTATTCAGTTACGCTTGTACGTTAAGGCGAATGCTTCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCA

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; AmpR – gene encoding the protein responsible for ampicillin resistance.
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.
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; AmpR – gene encoding the protein responsible for ampicillin resistance.
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.
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<sup>R</sup> – gene encoding the protein responsible for ampicillin resistance.
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance.
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; AmpR — gene encoding the protein responsible for ampicillin resistance; KmR — gene encoding the protein responsible for neomycin/kanamycin resistance.
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance.
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance.
Supplementary Figure S11. Plasmid pSN16K. The plasmid map 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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance.
Supplementary Plasmid pSN5S. 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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; sacB – gene encoding the protein responsible for sucrose sensitivity.
A

pSN8KS

7,584 bp

B

> pSN8KS
CACCAGGCACGCCTTAACTGCGCAGCCTACAGGCGGATTTAGTAGGGATACCGACGTTTTTCCAGTACGAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCACGCTGGCAGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGATGAAGCTGGCGGAGTTAGTCCAGGGTTATTTGTACCGTCAAGGTTACATTGCCACCCCAGATTTAACTACGCTGCGAGTCCGTACCATTGGCGATCGGGCCTATCTCACCATTAAGGGTAAAAATGCCAGCATTGCCCGCTTGGATTTGAGTATGAAATTCTTGCAGTGGAAGCCAATTAAATTTTGACAAGACTCTGTTCACCGCCCCTGATTGAAAATATCGTTATTGCCTCGATTACCATGGTAAAACCTGA
GAAGTAGACGAGTTTTTGGGGGATAACCAGGGTCTAATTTTAGCGGAAGTGGAATTAACCTACACTGGTGAAAAAATAAGTCTACTTCCCTGGATCGGGGAAGAGGTAACGGATGATGCCCGCTACCTGCTGGCCGTCGGCCAGGGCTACAAAATCACGGGCGTCGTGGACTATGAGCACGTCCGCGAGGGCGTCCCGGAAAACGATTCCGAAGGCCAACCTTTCATAGAAGGCGGCGGTGGAATCGAAATCTCGTGATGGCAGGTTGGGCGTCGCTTGGTCGGTCATTTCGCTCGGTACCATCGGCATTCTTTTTTGCGTTTT
TATTTGTTAACTGTTAATTGTCCTTGTTCAAGGATGCTGTCTTTGACAACAGATGTTTTCTTGCCTTTGATGTTCAGCAGGAAGCTCGGCGCAAACGTTGATTGTTTGTCTGCGTAGAATCCTCTGTTTGTCATATAGCTTGTAATCACGACATTGTTTCCTTTCGCTTGAGGTACAGCGAAGTGTGAGTAAGTAAAGGTTACATCGTTAGGATCAAGATCCATTTTTAACACAAGGCCAGTTTTGTTCAGCGGCT
TGTATGGGCCAGTTAAAGAATTAGAAACATAACCAAGCATGTAAATATCGTTAGACGTAATGCCGTCAATCGTCATTTTTGATCCGCGGGAGTCAGTGAACAGGTA CCATTTGCCGTTCATTTTAAAGACGTTCGCGCGTTCAATTTCATCTGTTACTGTGTTAGATGCAATCAGCGT TTTCATCACTTTTTTCAGTGTGTAATCATCGTTTAGCTCAATCATACCGAGAGCGCCGTTTGCTAACTCAGCCGTGCGTTTTTTATCGCTTTGCAGAAGTTTTTGACTTTCTTGAC GGAAGAATGATGTGCTTTTGCCATAGTATGCTTTGTTAAATAAAGATTCTTCGCCTTGGTAGCCATCTTCAGTTCCAGTGTTTGCTTCAAATAC TAAGTATTTTGCCCTTTATCTCTACTAGTGAGTATGAGATCTCAGGTTATGAGGAAGGACAGGACAATATTATTTTTTGCTGTCAGGGATTTGCAGCATATCATGGCG
TGTAATATGGGAAATGCCGTAT GTTTCCTTATATGGCTTTTGGTTCGTTTCTTTCGCAAACGCTTGAGTTGCGCCTCCTGCCAGCAGTGCGGTAGTAAAGGTTAATACTGTTGCTT GTTTTTGCAAACTTTTTGATGTTCATCGTTCATGTCTCCTTTTTTATGTACTGTGTTAGCGGTCTGCTTCTTCCAGCCCTCCTGTTTGAAGATGGCAAGTTAGTTACGCACAATAAAAAAAGACCTAAAATATGTAAGGGG
TGACGCCAAAGTATACACTTTGCCCTTTACACATTTTAGGTCTTGCCT
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; sacB – gene encoding the protein responsible for sucrose sensitivity.
sensitivity. for neomycin/kanamycin resistance; encoding the protein responsible for ampicillin resistance; Km
N10.5
is depicted in
Supplementary Figure
GGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCA
AACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTA
GCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATC
ATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGG
AATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGAA
TCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACA
TCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAA
AAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCA
AATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGG
ATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACT
AGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTC
TGCGCAAC
GAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTT
CTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCC
TAATCAGTGAGGCACCTATCTCAGCGATCTGTCT
ATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAA
AAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTG
GCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAA
CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGT
GTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTA
CTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCT
CCCTGACGAGCATCACAA
ATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCC
TCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGG
TTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCG
CACACAACATACGAGCCGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCGC
CATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTC
ACCAAAACCAGGCTTGGAAAATACCCGTCCGCAAAGGGGCATTAAATCCACCGGGCGACTCGACCATATG
TGAAATCCCCTGACTTTTTCCGAGTTGGGGGACAAAATCAACTAATTTGGGCGACGATTTTTTCTGCTATCTGGCGATCGCCATGTTTGTCTAACTTC
AGGTGTCAGATAGCATTTAGATCGTAAAAGCATACGGCATGAACAACCATGAACGGCTTCCCAGAGGTTAACCCCAGGTTTATGGTTTACCAAA
TGTAATCCCCATACTCATGCAAAAGACATTAGCCTTGGAAGCTCC
S14. and the FASTA formatted sequence in
AAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTT
CTGAGAGAATGCCTGAAAAGAACCACTAAGTCAAATTATTTGGATTAAGTTTTATTAAGTCAACGG
ATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGG
CTGATGAGATCCTCCTACTACGAGCTCTTGGTGTAGGTCGTTCGCTTGGTGGTCGAATGGGCAGG
GCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCC
– gene encoding the protein responsible for sucrose
gene encoding the protein responsible
of

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; AmpR – gene encoding the protein responsible for neomycin/kanamycin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; sacB – gene encoding the protein responsible for sucrose sensitivity.
Supplementary Figure S15. Plasmid pSN15KS. The plasmid map with the main features highlighted in **A** and the FASTA formatted sequence in **B**. N15.3 – 3' homologous region for site N15; TT – double transcriptional terminators; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; sacB – gene encoding the protein responsible for sucrose sensitivity.
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.
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; gfp – sequence encoding the reporter GFP.
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; gfp – sequence encoding the reporter GFP.
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; gfp – sequence encoding the reporter GFP.
**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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; gfp – sequence encoding the reporter GFP.
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; gfp – sequence encoding the reporter GFP.
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; Ppsba2* – synthetic minimal constitutive promoter based on the native promoter of psba2 gene; gfp – sequence encoding the reporter GFP.
Supplementary Figure S23. Plasmid pSN8K.Cgfp. The plasmid map with the main features highlighted is 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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; Psba2* – synthetic minimal constitutive promoter based on the native promoter of psba2 gene; gfp – sequence encoding the reporter GFP.
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; Ppsba2∗ – synthetic minimal constitutive promoter based on the native promoter of psba2 gene; gfp – sequence encoding the reporter GFP.
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; Ppsba2* – synthetic minimal constitutive promoter based on the native promoter of psba2 gene; gfp – sequence encoding the reporter GFP.
>pSN16K_Cgfp
CAACCGCGCCGCTTAATGCGCGCCTAACCAGGGCGCCGCTATGCCGATCAGGCGAAGGGCGATGGTGGGCCGCCCTCTTCG
CTATATCCAGCGCTGAGGCGAAATGCGCGCTACAGGGCGCGTCG
CTTGGGATGGTGATGGTGGGTAAAGCTTCATTGTCCATGCGCCGGAGCTTGACGGTAAAATCCGTGTCCTGGGGCCAATCATTGGGAACGCTCAC
CAGATACCTGGCGTTAAGTCGAGGATATCCCCAACATCCACCATACCTAAGATCGCATGAGAACGTGAACCGCCGCCGTCGTATTTG
CTCTTTTGTTTGATAGAAAATCATAAAAGGATTTGCAGACTACGGGCCTAAAGAACTAAAAAATCTATCTGTTTCTTTTCATTCTCTGTATTTT
TTATATGTTTTCTGTGATGGGCGAATAGTGTCCTTTTTTAACATAATACATTAATATCCTAATACATATGGAAGGACGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTTGCCGCCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGAACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCTGGCCCCGGCACT
TCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCTTGGAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAA
GCAGCCGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCA
ATCATGCGAAACGATCCTCATCCTGTCTCTTGATCAGATCTTGATCCCCTGCGCCATCAGATCCTTGGCGGCAAGAAAGCCATCCAGTTTACTTT
TGCAAGGGCTTCCCAACCTTACCAGAGGGCGCCCCAGCTGGCAATTCCGGTTCGCTTGCTGTCCATAAAACCGCC
CAGTCTTGGGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCACAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCGGTTATGGTGTTCAATGCTTTGCGAGATACCC
AGATCATATGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATATT
TTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTA
AAAGAAGATGGAAACATTCTTGGACACAAATTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGG
ATCA
AAGTTAACTTCAAAATTAGACACAACATTGAAGATGGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGT
CCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTA
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; AmpR – gene encoding the protein responsible for ampicillin resistance; KmR – gene encoding the protein responsible for neomycin/kanamycin resistance; Ppsba2* – 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 $OD_{730} \approx 0.8–0.9$. 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).
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 (SNnKS). *Synechocystis* wild-type and mutants were grown in agar plates containing BG11 medium (BG11), BG11 medium supplemented with 100 µg mL⁻¹ kanamycin (BG11 + Km¹⁰⁰) 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 (SNrK.gfp) or a module with *gfp* under the control of the synthetic constitutive promoter $P_{\text{psba2}^+}$ (SNrK.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 OD₇₉₀. 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.