Figure S1: Screening for putative Gen1 episome-containing plants. (a) Schematic representation of Gen1 (~12.7 kb) and pSSC (~11.6 kb) vector integration in the plastome. The trnI/trnA and ndhG/ndhI homologous arms are indicated in Gen1 and pSSC, respectively. A dual selection cassette is also indicated: Prom-SD (P): rrr promoter along with a Shine-Dalgarno sequence (gray); aadA: spectinomycin resistance gene (yellow); 5'UTR: 5’ untranslated region (blue); smGFP: gene encoding the soluble monomeric green fluorescent protein (green); and 3'UTR (T): 3’ untranslated region (light gray). The kanamycin (KanR) or spectinomycin (SpcR) resistance gene are indicated in Gen1 and pSSC backbones, respectively. Location of primers used to check integration in the trnI/trnA (black arrows) and ndhG/ndhI (purple arrows) along with primers to check KanR (red arrows) and SpcR (blue arrows) genes are indicated. (b) PCRs to check vector integration in Gen1 and pSSC lines. Gen1-integrating lines originated with another version of the transgene cassette are indicated with asterisk (*). Per each construct, PCR samples from lines 1-15 are shown. DNA bands of 2.8 and 2.6 kb indicate integration of Gen1 and pSSC, respectively. DNA bands of 0.46 and 0.44 kb indicate wild-type (WT) IR and SSC regions of the plastome, respectively. The presence of both transgenic and wild-type bands indicates that all Gen1 and pSSC lines at the first vegetative generation are heteroplasmic. Wild-type (WT) samples, blanks (B) and DNA markers (M; kb) are shown. (c) Presence of backbone vector in different transplastomic lines. PCRs using primers for KanR (0.8 kb) indicate the presence of the backbone vector in leaf samples of two putative Gen1 lines containing the episome (Gen1; line 2 and 7). PCRs using primers for SpcR confirm the absence of backbone in pSSC-integrating plants. Positive (Gen1 plasmid; p) and negative (Gen1-integrating lines) controls along with wild-type (WT) samples, blanks (B) and DNA markers (M; kb) are shown.
Figure S2: Phenotype of transgenic lines and wild-type controls. (a) Images showing 2-week-old *in vitro* transgenic lines along with wild-type controls. pSSC and Gen1-integrating lines, along with a plant containing εGen1 are shown. (b) Confocal images showing smGFP localization into the chloroplast stroma of leaf mesophyll cells from transgenic lines in A. smGFP (green), chlorophyll (Chl., red), bright-field (BF; gray) and merged images are indicated. Scale bars: 10 mm (a); 20 µm (b).
Figure S3: Characterization of episomal lines containing eGen1. (a) Schematic representation of vector rearrangement in Gen1 (~12.7 kb) integrating and eGen1 (~10 kb) containing lines. In eGen1-containing lines the selection cassette is integrated in an unpredicted plastome site. The trnI/trnA homologous arms, the kanamycin resistance gene (KanR) of the backbone and cassette genetic modules are indicated. The cassette comprises: Prom-SD (P): rrn promoter along with a Shine-Dalgarno sequence (gray); aadA: spectinomycin resistance gene (yellow); 5’UTR: 5’ untranslated region (blue); smGFP: gene encoding the soluble monomeric green fluorescent protein (green); and 3’UTR (T): 3’ untranslated region (light gray). Location of primers used to check integration in the trnI/trnA region (black arrows), KanR (red arrows), aadA (yellow arrows) and smGFP (green arrows) are indicated. (b) PCRs showing vector rearrangement (Int.) in the IR (trnI/trnA) region of eGen1-containing and Gen1-integrating lines, respectively. Two independent lines of both genotypes are indicated (1 and 2). Sample 2 corresponds to the eGen1-containing line 2 (Figure S1) used in this study. DNA-bands at 2.8 kb indicate transgenes integration into the IR (trnI/trnA) site, whereas the presence of 0.46 kb bands indicate wild-type IR regions. (c-f) PCRs for smGFP (0.72 kb), aadA (0.79 kb), KanR (0.8 kb) and rbcL fragment (0.22 kb) are also indicated. Wild-type samples and blanks along with DNA molecular markers (kb) are shown.
Figure S4: PCR characterization of eGen1 plasmids extracted from leaf tissue of episome-containing lines. (a) Schematic representation of Gen1 (~12.7 kb) and eGen1 (~10 kb) plasmids. The trnI/trnA homologous arms, the kanamycin resistance gene (KanR) of the backbone and genetic modules composing the cassette are indicated. The cassette comprises: Prom-SD (P): rrr promoter along with a Shine-Dalgarno sequence (gray); aadA: spectinomycin resistance gene (yellow); 5'UTR: 5' untranslated region (blue); smGFP: gene encoding the soluble monomeric green fluorescent protein (green); and 3'UTR (T): 3' untranslated region (light gray). Location of primers used to check the presence of the full-length cassette inserted in between arms (black arrows), trnI arm (gray arrows) trnA arm (blue arrows), KanR (red arrows), aadA (yellow arrows) and smGFP (green arrows) are indicated. (b) PCRs using primers for the trnA and trnI homologous arms along with primers external of the dual-selection cassette were used to characterize the eGen1 plasmid extracted by back transformation into E. coli. Plasmids extracted from two independent colonies (1 and 2) are indicated. DNA bands of 4.3 and 2.5 kb at the same molecular weight of the positive control, Gen1, indicate the presence of full-length homologous arms in both eGen1 plasmids. On the contrary, the presence of lower-molecular weight bands of 0.46 kb rather than 2.8 kb (Gen1) indicate removal of the dual selection cassette in eGen1. (c) PCRs using primers for smGFP (0.72 kb), aadA (0.79 kb) and KanR gene (0.8 kb) confirmed the absence of the selection cassette and the presence of the backbone vector in eGen1. The Gen1, was used as positive control for comparison of the molecular weight of DNA bands. The negative controls (blanks) and DNA molecular markers (kb) are also indicate in the gels. These results have been confirmed by sequencing analysis of the entire eGen1 plasmid.
Figure S5: Determination of episome/plastome ratio of eGen1-containing lines at the 3rd round of tissue culture. (a) The vector rearrangement in plants containing eGen1 (~10 kb) leads the formation of the episome and unpredicted cassette integration. The trnI/trnA homologous arms, the kanamycin resistance gene (KanR) of the backbone and genetic modules of the selection cassette are indicated. The cassette comprises: Prom-SD (P): rrn promoter along with a Shine-Dalgarno sequence (gray); aadA: spectinomycin resistance gene (yellow); 5’UTR: 5’ untranslated region (blue); smGFP: gene encoding the soluble monomeric green fluorescent protein (green); and 3’UTR (T): 3’ untranslated region (light gray). Location of primers used to check KanR (red arrows), aadA (yellow arrows) and smGFP (green arrows) are indicated. (b) PCRs for KanR (0.8 kb), rbcL fragment (0.22 kb), smGFP (0.72 kb), and aadA (0.79 kb) using DNA samples extracted from two independent eGen1-containing lines (1 and 2) at the third round of tissue culture. The second eGen1-containing line 2 was the line selected for further study (Figure S1). The PCR profiles confirmed that eGen1 was stable at this developmental stage. Wild-type controls, blanks and molecular markers (kb) have been included. (c) Graph summarizing the ratio of copy number of episome vs plastome (copy n. episomal/plastome) in genomic DNA preparations of the selected eGen1-containing line determined by qPCR analysis from leaf tissue of transgenic plants and wild-type controls at the third round of tissue culture. Results are expressed as mean ± standard deviation (sd) of a total of 3 biological and 4 technical replicates per each biological replicate.
(a) 

2nd round (in vitro) 

Unspecific integration 

~ 0.79 kb ~ 0.72 kb 

(b) 

2 weeks (soil) 

4 weeks (soil) 

7 weeks (soil) 

10 weeks (soil) 

Markers: Marker, Gen1, Wild-type, Blank 

Kb: 0.8, 0.22, 0.72, 0.79 

Gen1 ~ 10 kb 

KanR ~ 0.8 kb 

aadA smGFP 

KanR 

rbcl 

smGFP 

aadA 

Unspecific integration 

~ 0.72 kb ~ 0.79 kb
Figure S6: Stability of e-Gen1 episome at different plant developmental stages. (a) The vector rearrangement in e-Gen1-containing plants leads to the formation of the episome (~10 kb) and unpredicted cassette integration. The trnI/trnA homologous arms, the kanamycin resistance gene (KanR) of the backbone and genetic modules of the dual selection cassette are indicated. The cassette comprises: Prom-SD (P): rrn promoter along with a Shine-Dalgarno sequence (gray); aadA: spectinomycin resistance gene (yellow); 5’UTR: 5’ untranslated region (blue); smGFP: gene encoding the soluble monomeric green fluorescent protein (green); and 3’UTR (T): 3’ untranslated region (light gray). Location of primers used to check KanR (red arrows), aadA (yellow arrows) and smGFP (green arrows) are indicated. (b) PCRs for KanR (0.8 kb), rbcL fragment (0.22 kb), smGFP (0.72 kb), and aadA (0.79 kb) using DNA samples extracted from e-Gen1-containing lines at the indicated developmental stages are shown. The PCR profiles confirmed that e-Gen1 (Figure S1) is stable throughout all plant developmental stages. Wild-type samples (WT), blanks and molecular markers (kb) have been included. (c) Representative plants at the second round of tissue culture (2nd round in vitro) grown on potting mix for 4, 7 and 10 weeks (anthesis); e-Gen1-containing lines along with wild-type control plants (WT). Scale bars = 5 cm.
Figure S7: Characterization of eGen1-containing lines originated from tubers.

(a) Schematic representation of vector rearrangement in Gen1 (~12.7 kb) integrating and eGen1 (~10 kb) containing plants originated from tubes. In eGen1-containing lines the selection cassette is integrated in an unpredicted plastome site. The trnI/trnA homologous arms, the kanamycin resistance gene (KanR) of the backbone and a cassette modules are indicated. The dual selection cassette comprises: Prom-SD (P): rrn promoter along with a Shine-Dalgarno sequence (gray); aadA: spectinomycin resistance gene (yellow); 5’UTR: 5’ untranslated region (blue); smGFP: gene encoding the soluble monomeric green fluorescent protein (green); and 3’UTR (T): 3’ untranslated region (light gray). Location of primers used to check integration in the trnI/trnA region (black arrows), KanR (red arrows), aadA (yellow arrows) and smGFP (green arrows) are indicated. (b) A second generation eGen1-containing plant regenerated from tubers of eGen1-containing line 2 plants grown on potting mix (line 2, Figure S1). (c) Bacterial colonies transformed with eGen1 contained in leaf tissue is shown in b. (d-h) PCRs for detection of KanR (0.8 kb), integration (2.8 or 0.46 kb bands, for integration or not, respectively), rbcL fragment (0.22 kb), smGFP (0.72 kb), and aadA (0.79 kb) using DNA samples extracted from eGen1-containing lines originated from tubers (T) or from tissue culture (L) are shown. A Gen1-integrating line along with wild-type controls, blanks and molecular markers (kb) are also shown. The PCR results confirmed the presence of eGen1 in plants originating from tubers. Scale bars = 10 mm (a-b).
Figure S8: Characterization of synplastomic Gen2-containing lines. (a) Schematic representation of vector rearrangement in transgenic plants integrating Gen1 (~12.7 kb), along with plants containing either the episome Gen1 (~10 kb), Gen2 (~12 kb) or Gen2Δ (~4.2 kb). In Gen1-containing line the selection cassette is integrated in an unpredicted plastome site, while in Gen2-containing lines the selection cassette is stably part of the episome backbone. The trnI/trnA homologous arms, the kanamycin resistance gene (KanR) of the backbone and genetic modules of the selection cassette are indicated. The cassette comprises: Prom-SD (P): rrn promoter along with a Shine-Dalgarno sequence (gray); aadA: spectinomycin resistance gene (yellow); 5’UTR: 5’ untranslated region (blue); smGFP: gene encoding the soluble monomeric green fluorescent protein (green); and 3’UTR (T): 3’ untranslated region (light gray). Location of primers used to check integration in the trnI/trnA region (black arrows), KanR (red arrows), aadA (yellow arrows), smGFP (green arrows) and an unique region of the full-length Gen2 episome (backbone type; purple arrows) are indicated. (b) PCR analysis of synplastomic Gen2-containing lines at the first cycle of tissue culture. DNA-bands of 2.8 kb indicate correct integration into the trnI/trnA plastome site in a Gen1-integrating plant used as positive control (Int.). Lower-molecular weight bands of 0.46 kb indicate wild-type IR regions of the plastome in all Gen2-containing lines. PCRs for rbcL fragment (0.22 kb), smGFP (0.72 kb), aadA (0.79 kb), KanR (0.8 kb) and backbone type (Type; 0.1 kb) to detect the presence of the full-length Gen2 are included. Gen2 (Gen21-3) or Gen2Δ-containing lines along with the two controls, Gen1-containing lines and Gen1-integrating lines are shown. Wild-type samples, blanks and molecular markers (kb) are also shown in the gels.
Figure S9: PCR characterization of \( e \text{Gen2} \) extracted from synplastomic plants. (a) Schematic representation of \( e \text{Gen1} \) (~12.7 kb), \( e \text{Gen1} \) (~10 kb) and \( e \text{Gen2} \) (~12 kb) plasmids. The \( trnI/trnA \) homologous arms, the kanamycin resistance gene (\( KanR \)) of the backbone and genetic modules forming the selection cassette are indicated. The cassette comprises: Prom-SD (P): rrn promoter along with a Shine-Dalgarno sequence (gray); \( aadA \): spectinomycin resistance gene (yellow); 5'UTR: 5' untranslated region (blue); \( smGFP \): gene encoding the soluble monomeric green fluorescent protein (green); and 3'UTR (T): 3' untranslated region (light gray). Location of primers used to check the presence of the full-length cassette inserted in between arms (black arrows), \( trnI \) arm (gray arrows) \( trnA \) arm (blue arrows), \( KanR \) (red arrows), \( aadA \) (yellow arrows) and \( smGFP \) (green arrows) are indicated. (b) PCRs for IR \( trnI/trnA \) homologous arms (short and long, respectively) along with the dual-selection cassette were used to characterize \( e \text{Gen2} \) extracted from synplastomic lines by back transformation into \( E. coli \). PCR bands of 4.3 and 2.5 kb at the same molecular weight of the positive control, \( e \text{Gen1} \), indicate the presence of homologous arms. The presence of lower-molecular weight bands of 0.46 kb rather than 2.8 kb (\( e \text{Gen1} \)) support correct transgene cassette location in the backbone. PCRs for \( smGFP \) (0.72 kb), \( aadA \) (0.79 kb) and \( KanR \) gene (0.8 kb) confirmed the presence of both the backbone and transgenes. Two bacterial colonies containing \( e \text{Gen1} \) and \( e \text{Gen1} \) were isolated for comparison. The negative controls (blanks) and DNA molecular markers (kb) are also indicate in the gels. These PCR results were confirmed by sequence analysis of the entire \( e \text{Gen2} \).
Figure S10: Southern blot profile of synplastomic eGen2-containing lines at the 2nd round of synplastomic plants. (a) Schematic representation of eGen2 (~12 kb) plasmids. The trnI/trnA homologous arms, the kanamycin resistance gene (KanR) of the backbone and genetic modules of the dual selection cassette are indicated. The cassette comprises: Prom-SD (P): rrn promoter along with a Shine-Dalgarno sequence (gray); aadA: spectinomycin resistance gene (yellow); 5’UTR: 5’ untranslated region (blue); smGFP: gene encoding the soluble monomeric green fluorescent protein (green); and 3’UTR (T): 3’ untranslated region (light gray). Location of restriction enzymes used for Southern blots, KasI/HindIII and FseI/FspI, and predicted size of DNA fragments are indicated. The KasI/HindIII and FseI/FspI fragments were detected by a ~0.5 kb probe designed on trnI/trnA (red bar) or KanR (blue bar), respectively. (b) Southern blot analysis performed using either an IR or KanR probe and leaf total DNA preparations extracted from eGen2-containing lines 1-3 and a line harboring eGen2Δ at the second round of transplastomic lines. Genomic DNA samples from eGen1-containing and Gen1-integrating lines along with wild-type plants were used as a comparison. Molecular weight of DNA fragments (kb) are indicated in the blots.
Figure S11: Transgene expression in Gen2-containing lines. RT-PCRs using cDNA preparations from Gen2, Gen1, and Gen1-integrating line along with wild-type controls and blanks have been included. A pair of primers specific for smGFP and aadA were used to detect the full-length cDNAs (0.72 and 0.79 kb, respectively). PCRs for rbcL and ef1 genes were used as loading controls (0.1 kb DNA bands) for the plastome and nuclear genome. Molecular weight markers (kb) are shown in the gel.
Figure S12: PCRs on bacteria colonies transformed with \( e \text{Gen2} \) contained in leaf tissue. (a) Schematic representation of \( e \text{Gen2} \) episome (~12 kb) and the \( e \text{Gen2}^\Delta \) short form (~4.2 kb) which contain a large deletion of the \( \text{trnI/trnA} \) homologous region (dotted line). In both \( e \text{Gen2} \) episomes the selection cassette is stably part of the episome backbone. The \( \text{trnI/trnA} \) homologous arms, the kanamycin resistance gene (\( \text{KanR} \)) of the backbone and genetic parts of the selection cassette are indicated. The cassette comprises: Prom-SD (P): rrr promoter along with a Shine-Dalgarno sequence (gray); \( \text{aadA} \): spectinomycin resistance gene (yellow); 5’UTR: 5’ untranslated region (blue); \( \text{smGFP} \): gene encoding the soluble monomeric green fluorescent protein (green); and 3’UTR (T): 3’ untranslated region (light gray). Location of primers used to check the presence of a \( \text{trnI/trnA internal} \) fragment (black arrows) and the \( \text{aadA} \) gene (yellow arrows) are indicated. (b) For each \( e \text{Gen2} \)-containing line (\( e \text{Gen2}_2 \) or \( e \text{Gen2}_3 \)), a total of ten \( E. \text{coli} \) colonies (1-10) were tested. The presence of the \( e \text{Gen2} \) backbone in all bacterial samples was confirmed by PCR-positive products for the \( \text{aadA} \) gene (0.79 kb). The presence of the homologous region of full-length \( e \text{Gen2} \) was detected by using primers for an internal region of 0.46 kb (H. arms). Asterisk (*) indicate bacterial samples from \( e \text{Gen2} \)-containing line 2, which contained the \( e \text{Gen2}^\Delta \) form (~50% of total colonies). Blanks and molecular markers (kb) are shown in the gels.
Figure S13: Stability of eGen2 at different plant developmental stages. (a) Schematic representation of eGen2 full-length (~12 kb), eGen2Δ short-form (~4.2 kb) and eGen1 (~10 kb) episomes. In eGen1 the cassette is removed, while in both types of eGen2 episomes is stably part of the backbone. The trnI/trnA homologous arms, the kanamycin resistance gene (KanR) of the backbone and genetic modules composing the selection cassette are indicated. The cassette comprises: Prom-SD (P): rrn promoter along with a Shine-Dalgarno sequence (gray); aadA: spectinomycin resistance gene (yellow); 5'UTR: 5’ untranslated region (blue); smGFP: gene encoding the soluble monomeric green fluorescent protein (green); and 3'UTR (T): 3’ untranslated region (light gray). Location of primers used to check KanR (red arrows), aadA (yellow arrows) and smGFP (green arrows) genes, along with primers to check an unique region of the full-length eGen2 episome (backbone type; purple arrows) are indicated. (b) Molecular characterization of eGen2-containing lines at 1, 4, 7 and 10 (anthesis) weeks on potting mix. eGen2-containing lines (eGen21-3), along with eGen1-containing and the Gen1-integrating control lines are shown. Five plants (1-5) per each genotype have been used. Wild-type samples, blanks and molecular markers (kb) are also shown in the gels. PCRs for KanR (0.8 kb), backbone type (Type; 0.1 kb) to detect the presence of full-length eGen2, smGFP (0.72 kb), aadA (0.79 kb) and rbcL fragment (0.22 kb) are included. Asterisk (*) indicate the line able to harbor eGen2Δ until anthesis (10 weeks on potting mix).