Methods in Molecular Biology 2317

Springer Protocols

Pal Maliga *Editor*

Chloroplast Biotechnology

Methods and Protocols

Second Edition

Humana Press

Chapter 21

Rapid and Modular DNA Assembly for Transformation of Marchantia Chloroplasts

Eftychios Frangedakis, Kasey Markel, Susana Sauret-Gueto, and Jim Haseloff

Abstract

The bryophyte *Marchantia polymorpha*, has attracted significant attention as a powerful experimental system for studying aspects of plant biology including synthetic biology applications. We describe an efficient and simple recursive Type IIS DNA assembly method for the generation of DNA constructs for chloroplast genome manipulation, and an optimized technique for Marchantia chloroplast genome transformation. The utility of the system was demonstrated by the expression of a chloroplast codon-optimized cyan fluorescent protein.

Key words Marchantia, Chloroplast, DNA loop assembly, Transformation, Type IIS

1 Introduction

Marchantia polymorpha is the best studied liverwort and has a series of characteristics that makes it an ideal experimental system. It has a small size and simple morphology, grows rapidly and resiliently under laboratory conditions, and has a remarkable regenerative capacity. As a bryophyte, the dominant phase of the Marchantia life cycle is haploid. Genetic studies are made easier by its small gene families and lack of redundancy [1]. Marchantia can reproduce asexually by means of clonal propagules called gemmae, which provide a powerful platform for live-tissue microscopy. Marchantia is also one of the few land plant species for which chloroplast transformation is well established [2, 3]. All the above are making Marchantia a promising testbed for the development of chloroplast engineering applications.

Type IIS DNA assembly systems such as Golden Gate [4], MoClo [5], and Loop assembly [6] have revolutionized molecular cloning. These DNA assembly systems are based on Type IIS restriction endonucleases, which have the special property that

Pal Maliga (ed.), *Chloroplast Biotechnology: Methods and Protocols*, Methods in Molecular Biology, vol. 2317,
https://doi.org/10.1007/978-1-0716-1472-3_21,

they digest DNA at a fixed distance from their recognition site, and allow for the generation of arbitrary "sticky overhangs," the sequence of which is not determined by the restriction enzyme used. As a result of the lack of overlap between the recognition site and the sticky overhangs, the sequence of the overhangs (fusion sites) can be customized to allow DNA fragments to be joined in a specific and standardized way. There is no requirement for purification of individual DNA fragments and end-products are generated in a combined digestion–ligation one-pot reaction. Legitimate products contain no sites for the restriction endonuclease, while illegitimate products do, and can be recleaved during the reaction. Thus the reactions are highly efficient, driven to accumulate the intended product. Type IIS assembly systems provide excellent platforms for the generation of DNA constructs.

Loop assembly is a Type IIS method for DNA construction that employs a recursive strategy to greatly simplify the process of plasmid assembly [7]. It allows rapid and highly efficient production of large DNA constructs. Unlike other systems that require elaborate sets of vectors, Loop assembly requires only two sets of four complementary vectors. Loop assembly uses two Type IIS restriction enzymes, BsaI which generates four base 5′ overhangs and SapI which generates three base 5′ overhangs. Alternate use of BsaI and SapI and corresponding vector sets, allows efficient and parallel assembly of large DNA circuits (Fig. 1a). In a single step reaction with BsaI and T4 ligase, standardized DNA parts (Level 0) can be assembled into transcriptional units (TUs), called Level 1 parts. Then up to four TUs (Level 1 parts) can be assembled together in a single step reaction with SapI and T4 ligase into constructs with multiple TUs, called Level 2 parts. The recursive nature of Loop assembly (Fig. 1b) means that Level 2 parts can be digested with BsaI and ligated to combine up to sixteen TUs in a Level 3 construct, which could be then digested again with SapI and ligated to create a Level 4 part with up to sixty four TUs.

A common syntax has been developed for standardized composition of Level 0 DNA parts in Type IIS assembly systems [7]. The common syntax defines specific, ordered fusion sites for DNA parts depending on whether they are promoters, UTRs, coding sequences or terminators. The common syntax enables modular assembly of multiple DNA standard parts and allows community-based efforts for building and exchanging collections of DNA parts. The common syntax was agreed to by an international team of plant biologists [8] and a simplified version can be used for chloroplast applications (Fig. 2). All Level 0 DNA parts are cloned into a universal acceptor plasmid. Users can customize the library of Level 0 parts with DNA parts suitable for their applications.

We have developed a new version of the Loop assembly system for rapid and simple construction of DNA vectors for transformation of Marchantia chloroplast called OpenPlant kit [9]. This

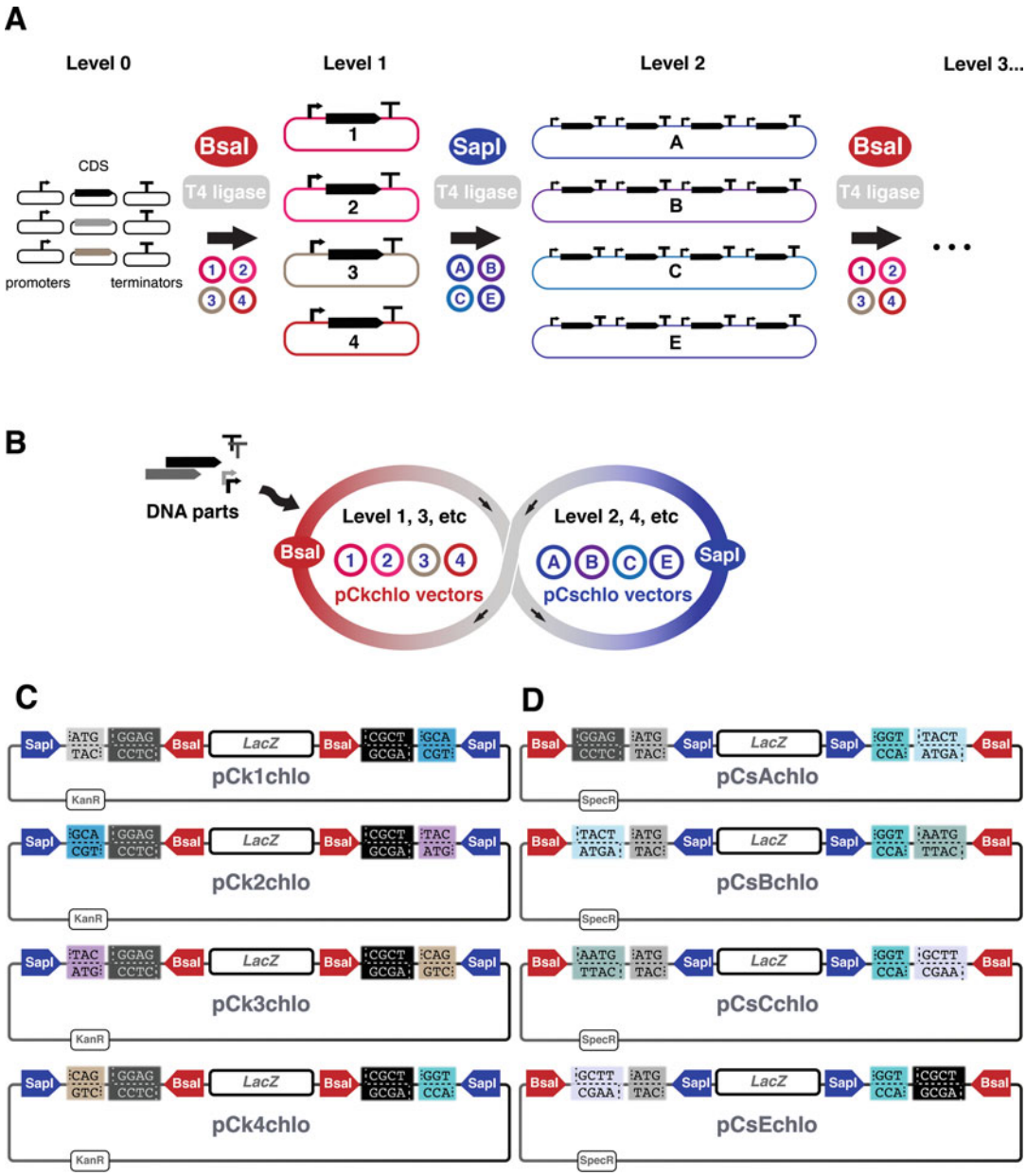

Fig. 1 Chloroplast Loop assembly. (**a**) Loop assembly overview. Level 0 DNA parts (for simplicity only promoter, coding sequence, and terminator genetic DNA parts are indicated) are assembled in Level 1 transcription units (TUs) into one of the four pCkchlo vectors, depicted with numbered circles, by BsaI-mediated Type IIS assembly (sequential restriction enzyme digestion and ligation reactions). Level 1 TUs are assembled to Level 2 multi-TUs into one the four pCschlo vectors, depicted with lettered circles, by SapI-mediated Type IIS assembly. This workflow is then repeated for higher level assemblies (Modified from [6]). (**b**) Graphic illustration of the recursive nature of Loop assembly**.** The recursive nature of Loop assembly means that Level 2 parts can be digested with BsaI and then be ligated to create a Level 3 part with up to 16 transcriptional units into a pCkchlo vector, which could be digested again with SapI and then be ligated to create a Level 4 part with up to 64 transcriptional units into a pCschlo vector and so on. pCkchlo vectors are used for odd number assemblies (Level 1, 3, etc.) and pCschlo vectors for even number assemblies (Level 2, 4, etc.) (Modified from [6]). (**c**) pCkchlo vectors, called pCk1chlo, pCk2chlo, pCk3chlo, and pCk4chlo,

includes the generation of a library of standardized DNA parts for gene circuitry and expression within Marchantia chloroplasts. The OpenPlant kit components are provided under an Open Material Transfer Agreement (OpenMTA) license for less restrictive sharing and open access [10] and have been deposited on Addgene [9]. The OpenMTA facilitates open exchange of biological materials, and was designed to support openness, sharing, and innovation in global biotechnology. In contrast to the widely used UBMTA, the OpenMTA allows redistribution and commercial use of materials, while still acknowledging the creators and promoting safe practices [10]. We hope that wide availability of the chloroplast Loop assembly system and associated Level 0 components will facilitate exploitation of plastid engineering.

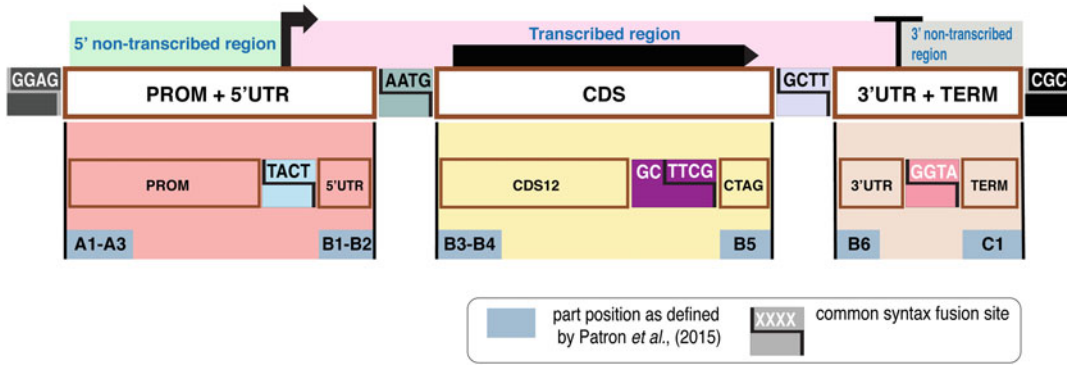

Fig. 2 Common syntax**.** The Patron et al. [8] common syntax was developed for eukaryotic plant nuclear gene structure with ten DNA part positions and 12 DNA part fusion sites defined. For chloroplast applications we have simplified the common syntax keeping only six positions and seven fusion sites. In particular we combined positions A1–A3: for promoter; B1–B2: for 5′ untranslated region; B3–B4: for coding sequence; and kept B5: for C-terminal coding region; B6: for 3′ untranslated region; and C1: for transcription terminator. For applications that a particular functional element is not needed, parts can span multiple fusion sites. For example, the fusion sites for a promoter are GGAG for the 5′ end and TACT for the 3′ end. A promoter can be combined with 5′ UTR into a single part with GGAG as the fusion sites for the 5′ end and AATG for the 3′ end (it should be noted that for the coding region fusion site, AATG, the three last nucleotides form the start codon of the gene coding sequence). Similarly, the 3′ UTR can be combined with the terminator with GCTT as fusion site are for the 5′ end and CGCT for the 3′ end

Fig. 1 (continued) contain divergent BsaI restriction sites and a specific set of 4 bp overhangs required for cloning Level 0 parts into them. They also contain convergent SapI restriction sites and a specific set of 3 bp overhangs, required for directing SapI-mediated Type IIS higher level assemblies. (**d**) pCschlo vectors, called pCsAchlo, pCsBchlo, pCsCchlo, and pCcEchlo, have divergent SapI restriction sites and a specific set of 3 bp overhangs to receive parts from pCkchlo plasmids. For higher level assemblies, pCschlo plasmids contain convergent BsaI sites and a specific set of 4 bp overhangs required for BsaI-mediated Type IIS. BsaI restriction sites are depicted with red arrows, SapI restriction sites are depicted with blue arrows. *KanR:* kanamycin bacterial resistance cassette, *SpecR:* spectinomycin bacterial resistance cassette, *LacZ: lacZα* cassette for blue–white screening

1.1 Plastid Transformation Vectors

We have created a new family of Loop assembly vectors, adapted for plastid transformation. These vectors have a backbone derived from pCAMBIA/pPZP vectors that contain a high copy number ColE1/pMB1/pBR322/pUC origin of replication [11]. They offer higher DNA yields during amplification in *E.coli* than the original Loop vectors. There are two sets of four vectors, one set for odd level number assemblies (i.e., Level 1, Level 3, etc) and another set for even level number assemblies (i.e., Level 2, Level 4, etc).

Vectors for odd level number assemblies (Fig. 1c) are named pCk1chlo, pCk2chlo, pCk3chlo and pCk4chlo (collectively pCkchlo, C: for CAMBIA k: for kanamycin, chlo: for chloroplast), they confer kanamycin resistance in bacteria and contain divergent BsaI restriction sites for odd level number Loop assemblies. pCk1chlo is a reduced derivative produced after removal of: the right and left border repeat from nopaline C58 T-DNA, and elements necessary for stability in Agrobacterium. The resulting vector has a size of 3.349 kb, reducing the total size of the plasmid DNA by 3.89 kb. pCkchlo vectors can be used to combine Level 0 DNA parts into a single TU, in a one-pot, one-step combined digestion–ligation reaction (as the example shown in Fig. 3). They can also be used as chloroplast transformation vector for Level 3 (or higher number, odd level) assemblies.

Chloroplast transformation vectors for even level number assemblies (Fig. 1c) are named pCsAchlo, pCsBchlo, pCsCchlo and pCsEchlo (collectively pCschlo, C: for CAMBIA s: for spectinomycin, chlo: for chloroplast). They confer spectinomycin resistance in bacteria and contain SapI divergent restriction sites for even level number Loop assemblies. pCsAchlo can be used as a chloroplast transformation vector for Level 2 (or higher number, even level) assemblies (as the example shown in Fig. 4). If less than 4 TUs are assembled, a TU can be replaced by spacer comprised of 200 arbitrary base pairs (bp). Spacer sequences were cloned in all four pCkchlo and pCschlo vectors and thus are available for each site, as needed.

All vectors have the *LacZ* cassette in a dropout configuration as a visual cloning selection marker. Bacterial colonies containing modified plasmids are white and colonies without digestion and replacement are blue.

For most chloroplast applications, Level 2 assemblies are sufficient. The first position and fourth position in the transformation vector is replaced by the homologous regions of the native chloroplast genome, 1–2 kb fragments, positioned upstream and downstream the integration region. This allows for customized targeted integration, via homologous recombination, of the second and third TU (Fig. 4).

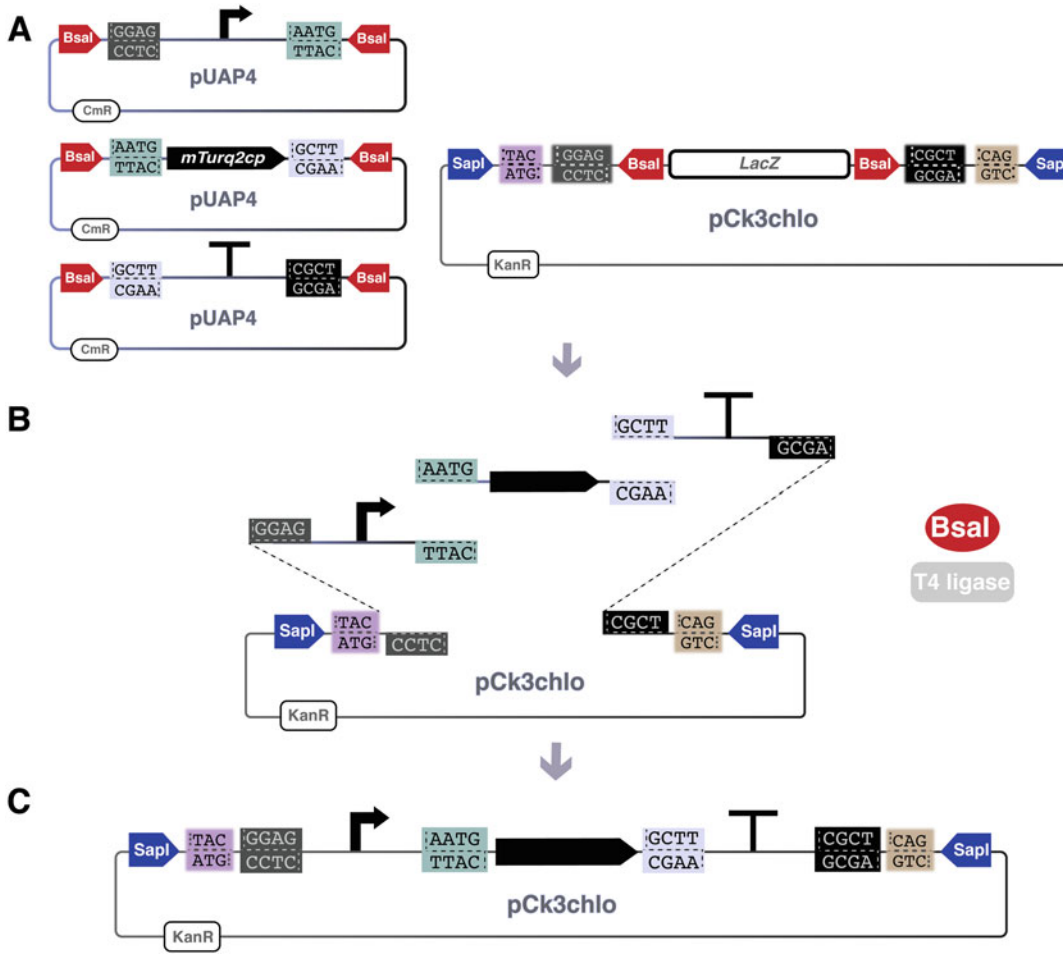

Fig. 3 Schematic representation of Level 1 assembly. Level 0 parts can be assembled in a Type IIS assembly reaction into a single TU in one of the four pCkchlo vectors. The Type IIS assembly reaction is composed of sequential restriction enzyme digestion and ligation reactions. In this example the Marchantia chloroplast DNA-dependent RNA polymerase operon promoter (*pMprpo*) (genome position: 5595–5739, GenBank accession no. MH635409) is assembled with the chloroplast codon optimized mTurquoise2 fluorescent protein coding sequence (*mTurq2cp*) [2] and the prokaryotic double terminator BBa_B0015 plus the rps16 terminator of the tobacco (*Nicotiana tabacum*) chloroplast genome [2] in one TU into the pCk3chlo vector. (**a**) The plasmids containing the Level 0 parts are placed in the same tube with the pCk3chlo plasmid, the BsaI and the T4 ligase enzymes. (**b**) First the Level 0 parts will be released from the pUAP4 backbone via BsaI mediated digestion revealing the necessary part overhangs for Level 1 assembly. Similarly the *LacZ* cassette will be released from the pCkchlo backbone revealing the necessary acceptor overhangs. (**c**) Then the Level 0 parts will be ligated into the pCkchlo vector creating the desired TU. Once the TU has been assembled, it loses the BsaI sites and thus cannot be further digested. After transformation of the Loop assembly Level 1 reaction into *E. coli* cells and plating on kanamycin antibiotic selection media all the remaining undigested pUAP4 vectors will be eliminated due to the different antibiotic selection marker they contain (chloramphenicol). BsaI restriction sites are depicted with red arrows, SapI restriction sites are depicted with blue arrows, the four nucleotide common syntax overhangs are shown in the pUAP4 vectors. *CmR:* chloramphenicol bacterial resistance cassette, *KanR:* kanamycin bacterial resistance cassette. *LacZ: lacZα* cassette for blue–white screening

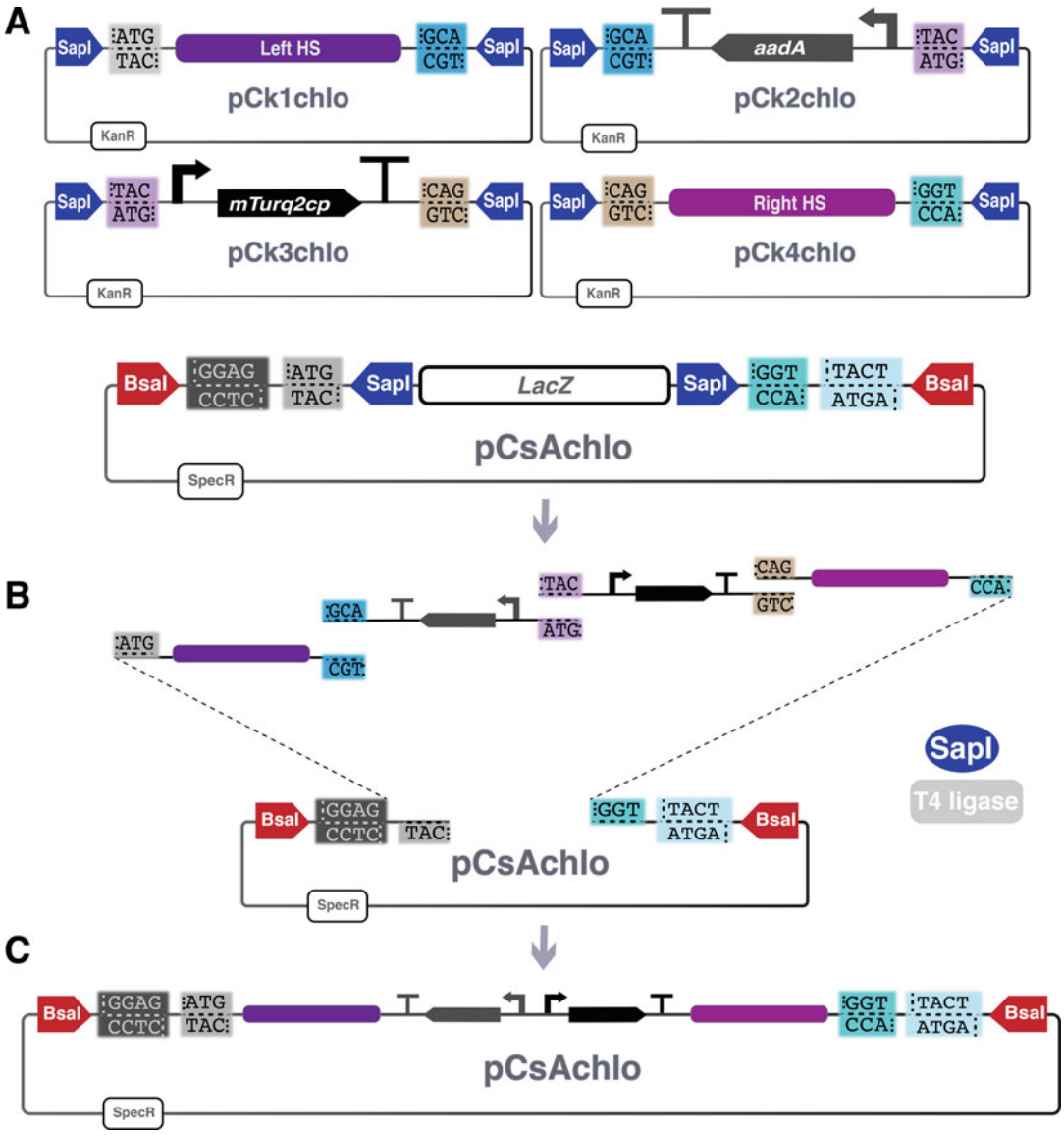

Fig. 4 Schematic representation of Level 2 assembly. The pCschlo vectors can be used to combine up to 4 TUs into a single Level 2 part in a one-pot, one-step SapI digestion–ligation reaction. (**a**–**c**) In this example, the left homologous sequence (HS) (genome position: 40,216–41,843, GenBank accession no. MH635409) and right homologous sequence (genome position: 41,847–43,622, GenBank accession no. MH635409) (regions upstream and downstream the *trnG–trnfM* intergenic region), are treated as "TUs" cloned into pCk1chlo and pCk4chlo respectively. They are assembled in a SapI-mediated Type IIS reaction with two more TUs into the pCsAchlo transformation vector: one TU, cloned into pCk2chlo, that corresponds to the spectinomycin resistance cassette [12] (in reverse orientation to avoid promoter activity overlap) and another TU, cloned into pCk3chlo, that corresponds to the mTurq2cp fluorescent protein expression cassette (same as Fig. 3). The spectinomycin resistance cassette is composed of the aminoglycoside adenyltransferase (*aadA*) gene coding sequence the tobacco (*N. tabaccum*) *rrn* promoter and the tobacco *psbA* terminator. After transformation of the Loop assembly Level 2 reaction into *E. coli* cells and plating on spectinomycin antibiotic selection media all the remaining undigested pCkchlo vectors will be eliminated due to the different antibiotic selection marker they contain (kanamycin). BsaI restriction sites are depicted with red arrows, SapI restriction sites are depicted with blue arrows. *KanR:* kanamycin bacterial resistance cassette, *SpecR:* spectinomycin bacterial resistance cassette, *LacZ: lacZα* cassette for blue–white screening

1.2 Chloroplast Transformation

Chloroplast transformation in Marchantia is achieved through particle bombardment of germinating spores [2, 3]. Millions of spores can be obtained from a single Marchantia cross [12], harvested in huge numbers and stored indefinitely in a cold, desiccated state. As a result, the tissue necessary for Marchantia chloroplast transformation is easily produced.

We have modified the protocol for plastid transformation of sporelings by adopting nanoparticles called DNAdel™ (Seashell Technology) as plasmid DNA carriers for the biolistic delivery into the chloroplast. DNAdel™ nanoparticles are 550 nm nanofabricated gold core carrier particles. According to the manufacturer, they have a modified surface for direct DNA immobilization to achieve high capacity loading and improved reproducibility of binding. The use of DNAdel™ reduces the time and labor required for loading of the plasmid DNA onto the microcarrier used for DNA delivery.

Once the desired Loop assembly construct is ready, it can be loaded onto the DNAdel™ nanoparticles, delivered to the chloroplast via biolistics (Fig. 5a–e) and integrated into a specific genome location via homologous recombination (as the example shown in (Fig. 5g–j). For the regeneration of Marchantia transplastomic plants there is no requirement for any special treatment other than selection of growth media containing antibiotic selection.

Overall, the strategy described in this chapter provides a simpler and faster method for both generation of chloroplast transformation constructs and DNA delivery. Only 30 min are required for the loading of DNA on the DNAdel™ nanoparticles compared to the 1.5 h required for loading of DNA on gold microcarriers in the previous protocol [2]. Successful transformants can be identified as early as 3 weeks after bombardment compared to 4–6 weeks reported in the previous protocol [2]. Chloroplast transformation construct generation can be achieved within a week. Notably, the DNA assembly method described in this chapter can be applied to other plant systems with only minor modifications.

2 Materials

2.1 DNA Assembly

1. 10× T4 DNA ligase buffer (#B0202S, New England Biolabs (NEB)), 1 mg/mL bovine serum albumin (BSA) (#B9000S, NEB), T4 DNA ligase (#M0202S, NEB), BsaI (#R0535S, NEB), 10 mM ATP (#A7699, SIGMA). 10× Tango buffer (BY5, Thermo Fisher Scientific), SapI (LguI) (#ER1931, Thermo Fisher Scientific), T4 DNA ligase (#EL0012, Thermo Fisher Scientific).
2. LB plates supplemented with 25 μg/mL chloramphenicol (#15353771, Fisher Scientific), LB plates supplemented with

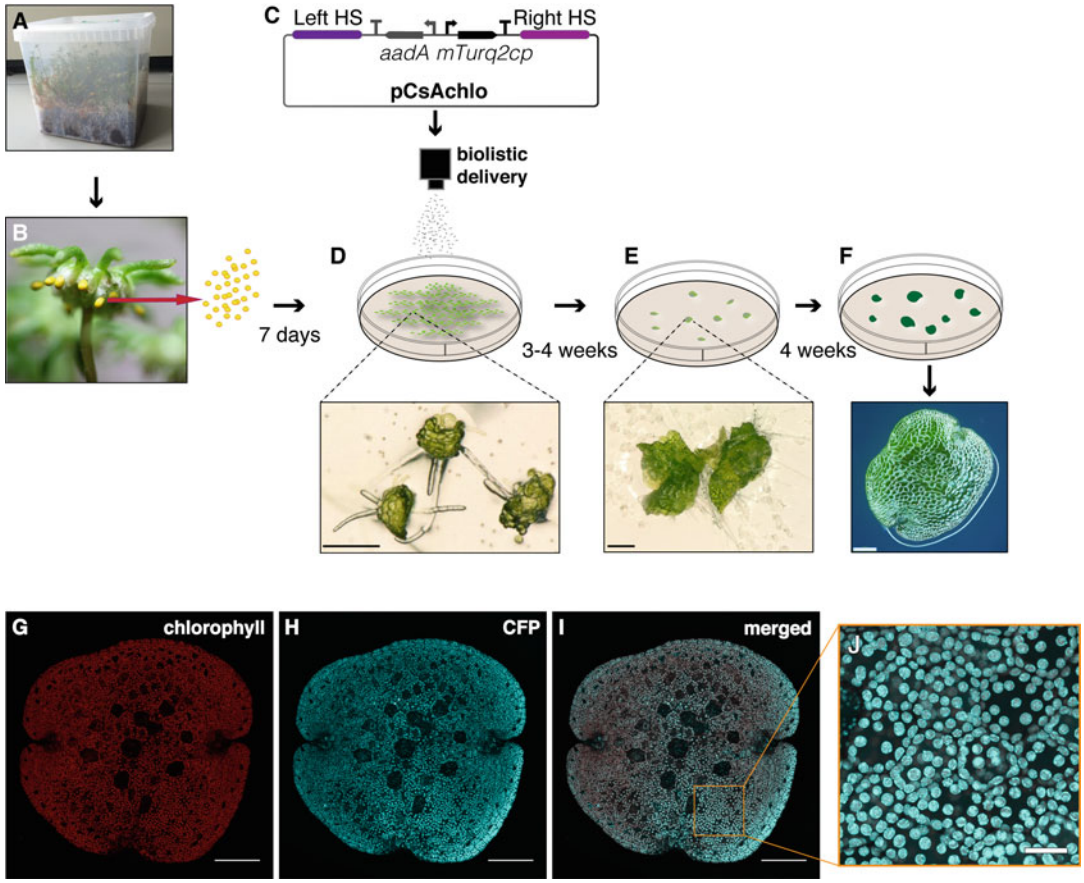

Fig. 5 Marchantia chloroplast transformation overview**.** (**a**) Microboxes are used to produce spores. (**b**) Spores are plated on 0.5× Gamborg B5 plus vitamins plates and incubated under continuous light for 7 days. (**c**, **d**) 7-day-old sporelings are bombarded with DNAdel™ nanoparticles coated with the desired Loop assembly construct (in this example same construct as in Fig. 4c). (**e**) After bombardment, plates are incubated on 0.5× Gamborg B5 plus vitamins plates supplemented with 500 μg/mL spectinomycin, and after 3–4 weeks successful transformants start to be visible. (**f**) Transformants are then transferred on a fresh 0.5× Gamborg B5 plus vitamins plate supplemented with 500 μg/mL spectinomycin. After the second round of selection (4 weeks), gemmae are produced and can be tested for homoplasmy by genotyping PCR. Scale bars **d**–**e**: 200 μm, **f**: 100 μm. (**g**–**j**) Microscopy images of Marchantia transplastomic gemma expressing the chloroplast codon optimized mTurquoise2 fluorescent protein (mTurq2cp) under the control of the native *Mprpo* promoter (same construct as Fig. 4). Images were taken with a Leica SP8 laser scanning confocal microscope. (**g**) Chlorophyll autofluorescence (excitation wavelength: 488 nm, emission window: 620–665 nm), (**h**) mTurq2cp (cyan fluorescent protein (CFP) excitation wavelength: 442 nm, emission window: 461–495 nm), (**i**) merged images and (**j**) higher magnification image showing mTurq2cp inside the chloroplasts of all cells. The transgene is integrated into the *trnG–trnfM* intergenic region. Scale bars: **g**–**i**: 100 μm, **j**: 20 μm

kanamycin 50 μg/mL (#11456412, Fisher Scientific) and LB plates supplemented with 100 μg/mL spectinomycin (#SB0901, Bio Basic). All LB plates are also supplemented with 40 μg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (#11680293001, Merck).

2.1.1 Agarose Gel Electrophoresis and Gel Extraction

1. 50× Tris acetate ethylenediaminetetraacetic acid (EDTA) solution, pH 8.3 (#20-6001-10, Severn Biotech).
2. QIAquick Gel Extraction Kit (#28706, QIAGEN).

2.1.2 PCR

1. Phusion High-Fidelity DNA Polymerase (2 U/μL) (#F530S, Thermo Fisher Scientific). 5× Phusion HF buffer (#F518L, Thermo Fisher Scientific).
2. Primers:
   UAP_F: 5′-CTCGAGTGCCACCTGACGTCTAAGAAAC-3′.
   UAP_R: 5′-CGAGGAAGCCTGCATAACGCGAAGTAATC-3′.
   pC_F: 5′-GCAACGCTCTGTCATCGTTAC-3′.
   pC_R: 5′-CAATCTGCTCTGATGCCGCATAGTTAAG-3′.

2.2 Growing Sporelings for Transformation

1. We use male and female accessions of *Marchantia polymorpha*, Cam-1 and Cam-2 respectively [13]. The Cam-1 and Cam-2 plastome sequences are identical, and have been submitted to GenBank with accession number MH635409 [9].
2. Culture media: 0.5× strength Gamborg B5 medium plus vitamins: 1.58 g/L Gamborg B5 media plus vitamins (#G0210.0050, Duchefa Biochemie) containing 1.2% (w/v) agar (#A20021-1000.0, Melford).
3. Selection media: For selection of chloroplast transformed plantlets, culture media were supplemented with spectinomycin dihydrochloride (#SB0901, Bio Basic) at a concentration of 500 μg/mL.
4. Gamborg B5 media plus vitamin composition.

Macroelements: $CaCl_2$ 113.23 mg/L, KNO_3 2500 mg/L, $MgSO_4$ 121.56 mg/L, NaH_2PO 130.44 mg/L, $(NH_4)2SO_4$ 134 mg/L. Vitamins: myoinositol 100 mg/L, nicotinic acid 1 mg/L, pyridoxine hydrochloride 1 mg/L, thiamine hydrochloride 10 mg/L Microelements: $CoCl_2 \cdot 6H_2O$ 0.025 mg/L, $CuSO_4 \cdot 5H_2O$ 0.025 mg/L, FeNaEDTA 36.70 mg/L, H_3BO_3 3 mg/L, KI 0.75 mg/L, $MnSO_4 \cdot H_2O$ 10 mg/L, $Na_2MoO_4 \cdot 2H_2O$ 0.25 mg/L, $ZnSO_4 \cdot 7H_2O$ 2 mg/L. Adjust pH to 5.8 using 5 M KOH.

2.2.1 Growth of Spores

1. 44 mm Jiffy-7 peat pellets (#32170142, Jiffy). Microboxes with green XXL+ filters (TP4000-TPD4000, SACO2, Belgium).
2. Spore sterilization solution is prepared by dissolving in water, Milton mini sterilizing tablets with active ingredient sodium dichloroisocyanurate (NaDCC, CAS: 2893-78-9), 19.5% w/w (Milton Pharmaceutical, UK).

2.3 Transformation and Sequencing

1. Two-four plates (per construct) with 7-day-old Marchantia germinating spores on 0.5× strength Gamborg B5 medium plus vitamins. Ethanol for molecular biology (#1085430250, Merck).
2. Plasmid DNA is prepared using the QIAGEN plasmid midi kit (#12143, QIAGEN).
3. Nanoparticles are DNAdel™ gold carrier particles size 550 nm, binding buffer and precipitation buffer (#S550d, Seashell Technology, info@crittertechnology.com).
4. Genotyping buffer: 100 mM Tris–HCl (#T60040–500.0, MELFORD), 1 M KCl (#P41000-2500.0, MELFORD) 1 M KCl, and 10 mM EDTA (#E58100-500.0, MELFORD) with pH 9.5.

2.4 Equipment

Microcentrifuge, 900 PSI rupture disks (#165-2257, Bio-Rad Laboratories), Macrocarriers (#165-2257, Bio-Rad Laboratories), Biolistic PDS-1000/He Particle Delivery System (#165-2258, Bio-Rad Laboratories), vortex, tweezers, 1.5 mL microfuge tube, Parafilm, 40 μM CellStar Cell Strainers (# 542040, Greiner Bio-One). Molecular sieves (#334286, SIGMA). Micro-pestle (#SIAL501ZZ0, SIGMA). Fluorescent lights: TL-D 58W/853 2F (Philips). Far red lights: Green Power LED HF (Philips). Ultrasonic Micro Cleaning Bath (#U50, ULTRAWAVE LIMITED, UK). Silica gel self-indicating (#S/0761/60, Fisher Scientific). Micropore tape (#1530-0, 3 M). Disposable plastic pipettes (#30-0135, CamLab). 50 mL Falcon tubes (#10788561 Fisher Scientific).

3 Methods

For the generation of a Marchantia chloroplast transformation construct using Loop assembly, the first step is the synthesis of Level 0 DNA parts. Level 0 DNA parts can be combined into a single TU in a Level 1 assembly that can then be combined into up to four TUs in a Level 2 assembly, which is sufficient for most applications (*see* **Note 1**).

3.1 DNA Assembly

3.1.1 Level 0 Reaction Protocol

For the generation of a Level 0 part the following steps should be followed:

1. Design primers as described in Fig. 6 to add the necessary overhangs for cloning into pUAP4 and also the overhangs that correspond to the common syntax. Briefly, primers should include, ordered from the 5′ terminus: (a) 3 random bp, (b) 7 bp SapI recognition sequence, (c) 5 bp that correspond to a partial BsaI recognition sequence, (d) one arbitrary base

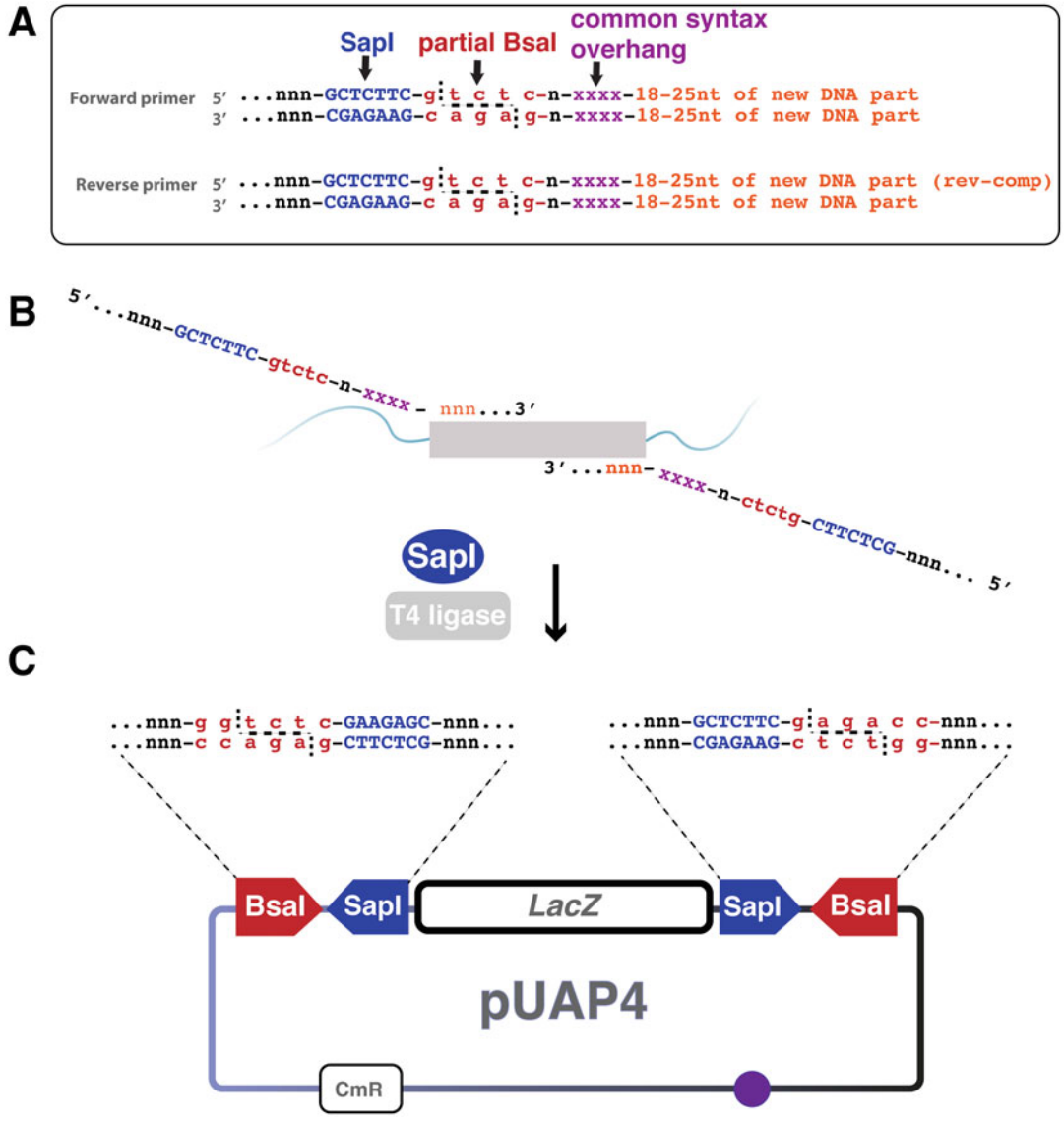

Fig. 6 Guide for Level 0 DNA part generation. For Loop assembly the universal acceptor plasmid is called pUAP4 and is a derivative of the pUAP1, an OpenMTA vector used for cloning of Level 0 part in the MoClo kit [7]. (**a**) The common syntax overhangs and also the necessary overhangs for Loop assembly can be added by PCR, using specially designed primers. Primers should include, ordered from the 5′ terminus, 3 random bp, then the 7 bp SapI recognition sequence (in blue), then 5 bp that correspond to a partial BsaI recognition sequence (in red), one random bp for spacing, then the common syntax overhang (in purple) followed by 18–25 bp of the DNA part to be amplified (in orange). (**b**) The DNA part should be amplified with a high fidelity DNA polymerase and (**c**) in a one-step SapI-mediated Type IIS assembly reaction, cloned into pUAP4. Once cloned into the pUAP4 the full BsaI recognition site is reconstituted allowing Level 1 assemblies. SapI restriction sites are depicted with blue arrows, CmR: chloramphenicol bacterial resistance cassette and LacZ: *lacZα* cassette for blue–white screening. Filled purple circle: ColE1/pMB1/pBR322/pUC origin of replication

for spacing, (e) the common syntax overhang followed by (f) 18–25 bp of sequences homologous to the DNA segment to be amplified.

2. If necessary, remove internal BsaI and SapI sites (*see* **Note 2**).
3. DNA parts are PCR amplified from the source DNA part (e.g., plasmid DNA or genomic DNA), using a high fidelity DNA polymerase such as Phusion. Use 10 ng of DNA if the template is plasmid DNA and 100 ng if the template is genomic DNA. The cycling conditions are: denaturation at 98 °C for 30 s; 35 cycles of denaturation at 98 °C for 10 s, annealing at primer annealing temperature for 30 s, and extension at 72 °C for 15 s/kb; and final extension at 72 °C for 10 min.
4. Run the PCR products on a 1.5% (w/v) agarose gel.
5. Gel extract the band that corresponds to the size of the amplified DNA part using a kit such as QIAquick Gel Extraction Kit.
6. Determine DNA concentration with spectrophotometry (Nanodrop).
7. Prepare aliquots of the DNA part at a concentration of 15 nM and of the pUAP4 acceptor vector at a concentration of 7.5 nM (*see* **Note 3**).
8. Set up a Type IIS assembly reaction into a 0.2 mL tube according to Table 1 to clone the amplified DNA part into pUAP4.
9. Mix well.
10. Place samples on a thermocycler and use the following program. Assembly: 26 cycles of 37 °C for 3 min and 16 °C for 4 min. Termination and enzyme denaturation: 50 °C for 5 min and 80 °C for 10 min.

Table 1
Level 0 reaction composition

Components	Volume (μL)
Sterile water	5
pUAP4	1
DNA part	1
10× Tango buffer (Thermo Fisher)	1
1 mg/mL bovine serum albumin (NEB)	0.5
T4 DNA ligase (5 U/μL) (Thermo Fisher)	0.25
10 mM ATP (SIGMA)	1
SapI (LguI) (5 U/μL) (Thermo Fisher)	0.25
Final volume	10

11. Transform 20 μL of chemically competent *E. coli* cells (transformation efficiency of 1×10^7 transformants/μg plasmid DNA) using 2 μL of the assembly reaction and then spread on LB agar plates containing 25 μg/mL chloramphenicol and 40 μg/mL X-gal.
12. Incubate O/N at 37 °C.
13. Select white colonies for sequencing. Loop assembly efficiency is 83–99% [6].
14. Confirm the presence of the correct insert with Sanger sequencing using the primers UAP_F and UAP_R and any additional DNA part specific primers.

3.1.2 Level 1 Reaction Protocol

1. Determine the concentrations of the DNA parts by spectrophotometry (Nanodrop).
2. Prepare aliquots for DNA parts to be assembled at a concentration of 15 nM and of the pCkchlo vector at a concentration of 7.5 nM (*see* **Note 3**).
3. Prepare Loop assembly Level 1 reaction master mix (MM) (*see* **Note 4**) according to Table 2, if four or fewer number of parts are assembled into a pCkchlo vector. For n reactions prepare a volume of MM to allow for $n + 1$ number of reactions (e.g., prepare a volume of 35 μL for six reactions).
4. Prepare plasmids mix for each reaction, by adding in a 0.2 mL tube, 1 μL of each DNA part aliquot, 1 μL of the pCkchlo vector and sterile water up to 5 μL. Mix well.
5. Add 5 μL of master mix to the 5 μL of plasmids mix, to a final volume of 10 μL. Mix well. If more than four DNA parts are to be assembled into a pCkchlo vector, reduce the water volume in the MM by 1 μL for each extra 1 μL of DNA part added in the plasmids mix.

Table 2
Level 1 master mix composition

Components	Volume (μL)
Sterile water	3
10× T4 ligase buffer (NEB)	1
1 mg/mL Bovine serum albumin (NEB)	0.5
T4 DNA ligase at 400 U/μL (NEB)	0.25
10 U/μL BsaI (NEB)	0.25
Final volume	5

6. Place samples in a thermocycler and use the following program: 26 cycles of 37 °C for 3 min and 16 °C for 4 min. Termination and enzyme denaturation: 50 °C for 5 min and 80 °C for 10 min.
7. Transform 20 μL of chemically competent *E. coli* cells (transformation efficiency of 1×10^7 transformants/μg plasmid DNA) using 2 μL of the Loop assembly reaction and then plate on LB agar plates containing 50 μg/mL kanamycin and 40 μg/mL X-gal.
8. Incubate O/N at 37 °C.
9. Colonies with white color are likely to contain the vector with the insert while blue color colonies will contain the empty vector.
10. Confirm the presence of the correct insert with Sanger sequencing using the primers pC_F and pC_R.

3.1.3 Odd Level from Level 3 Onwards Reaction Protocol

The protocol is same as Level 1 with the exception that number of plasmids is always 5 (4 Level 2 pCschlo plasmids and 1 pCkchlo acceptor vector). Thus, both the MM and the plasmids mix final volume is always 5 μL.

3.1.4 Even Level Reaction Protocol

Protocol is same as Level 1 with the exception of MM composition and antibiotic resistance of pCschlo vectors.

1. Determine DNA parts concentration with spectrophotometry (Nanodrop).
2. Prepare aliquots for DNA parts to be assembled at a concentration of 15 nM and of the pCschlo vector at a concentration of 7.5 nM.
3. Prepare the Loop assembly Even Level reaction MM according to according to Table 3.

Table 3
Even level master mix composition

Component	Volume (μL)
Sterile water	2
10× Tango buffer (Thermo Fisher)	1
1 mg/mL Bovine serum albumin (NEB)	0.5
T4 DNA ligase (5 U/μL) (Thermo Fisher)	0.25
10 mM ATP (SIGMA)	1
SapI (LguI) (5 U/μL) (Thermo Fisher)	0.25
Final volume	5

4. Transform 20 μL of chemically competent *E. coli* cells (transformation efficiency of 1×10^7 transformants/μg plasmid DNA) using 2 μL of the Loop assembly reaction and then plate on LB agar plates containing 100 μg/mL spectinomycin and 40 μg/mL X-gal.
5. Incubate O/N at 37 °C.
6. Select white colonies for sequencing.
7. Confirm the presence of the correct insert with Sanger sequencing using the primers pC_F and pC_R.

3.2 Spore Production

Marchantia spores can be obtained usually in three months using Microboxes. The lid of Microboxes has a specially designed filter that allows high gas exchange, limited dehydration and blocks the entry of contaminants such as fungi into the culture. We are using the TP4000 + TPD4000 Microboxes with a green XXL+ filter.

1. Put 15 "Jiffy 7" pellets in a Microbox, add 800 mL of water, close the lid and autoclave.
2. After autoclaving the Microbox with the "Jiffy 7" pellets, work in a flow hood.
3. Put at least one 10 mm × 10 mm thallus fragment or three gemmae per pellet using sterile tweezers.
4. Add another 150–200 mL of sterile water (if necessary), close the lid and place at 21 °C under continuous light with light intensity of 150 μmol/m^2/s.
5. After 1 month transfer plants to a chamber with a 16 h light/8 h dark regime, with light intensity of 150 μmol/m^2/s supplemented with far red light (peak emission: 700–750 nm, spectral photon flux density: 2400 μmol/m^2/s, distance from plants: 32 cm).
6. After approximately 4 weeks mature male and female reproductive organs are produced.
7. In a flow hood, open the lid of the Microbox, and using a pipette add 50–100 μL of sterile water on top of the male reproductive organ. After a few seconds you will be able to see the sperm as a cloudy exudate in the water drop.
8. For fertilization, transfer the drop with a disposable sterile plastic pipette on the top of the female reproductive organ. Usually 60–70 fertile female gametophores are produced in one Microbox which can produce several hundred mature sporangia.
9. After 1 more month mature sporangia should be visible and ready for collection. Place 1–2 mature sporophytes in an 1.5 mL centrifuge tube with five silica gel beads and store at

4 °C if the spores are to be used within 1–2 months. For longer storage, allow the sporophyte to desiccate for a week at 4 °C and then store at −80 °C.

3.3 Growing Sporelings for Transformation

1. Add one Milton tablet (*see* **Note 5**) into 25 mL of sterile water to prepare the sterilization solution.
2. Use four sporangia per transformation. A single sporangium contains several hundred thousand spores [12].
3. Add the sporangia into a 1.5 mL centrifuge tube.
4. Add 1 mL of sterilization solution into the tube and use sterile metal tweezers to crush the sporangia.
5. Place a 40 μM cell strainer into a 50 mL Falcon tube and pour the 1 mL suspension of crushed spore heads onto the filter and then wash the strainer with another 1 mL of sterilization solution.
6. Once filtered, aliquot the spore solution in 1.5 mL Eppendorf tubes (1 mL per tube) and incubate at room temperature for 10 min (the exposure to the sterilization solution should not exceed 25 min).
7. Centrifuge for 2 min at 15,800 × *g* at room temperature, discard the supernatant and resuspend the spores in sterilization solution (150 μL per transformation planned).
8. Spread 150 μL of resuspended spores on a plate containing growth media, leave to dry, seal the lid with micropore tape and place under continuous light at 21 °C 150 μmol/m^2/s for 7 days. Incubate with the bottom side up, which will prevent rhizoids from growing into the agar, making the removal of sporelings from the plate easier by simply using a sterile scalpel.

3.4 Transformation

3.4.1 Plasmid DNA Preparation

Prepare plasmid DNA using a kit such as QIAGEN plasmid midi kit. 15 μg of plasmid DNA are used for each construct to be transformed. Adjust sample concentration to 1 μg/μL with sterile water.

3.4.2 Nanoparticle Preparation

Before bombardment place the macrocarriers in the macrocarrier holders using forceps, place in a glass petri dish, wrap with aluminum foil and autoclave. Do the same for the rupture disks and stopping screens. In addition, place 10 mL absolute ethanol (100% EtOH) in a 50 mL Falcon tube sealed with parafilm in −20 °C (*see* **Note 6**).

1. DNAdel™ nanoparticles are supplied as a 50 mg/mL suspension in binding buffer. To dissociate any aggregates prior to use, agitate the suspension, sonicate briefly for 30 s using an ultrasonic water-bath sonicator, vortex for 5 s and repeat.
2. Use 0.5 mg of nanoparticles per transformation.

3. Dilute DNAdel™ nanoparticles into binding buffer to final concentration 30 mg/mL. For example, for three shots, mix 30 μL of 50 mg/mL DNAdel™ nanoparticles with 20 μL Binding Buffer (Seashell Technologies) into a 1.5 mL centrifuge tube.
4. Add 1.5–2 μg of plasmid DNA per shot planned. For example for three shots add 4.5–6 μg of plasmid DNA.
5. Add an equal volume of Precipitation Buffer (Seashell Technologies) (total volume of DNAdel™ nanoparticles plus volume of Binding Buffer plus volume of plasmid DNA), vortex and incubate at room temperature for 3 min.
6. Centrifuge at 8000 × *g* for 10 s, discard supernatant, and wash the DNA coated DNAdel™ nanoparticles with 500 μL ice cold 100% EtOH.
7. Centrifuge at 8000 × *g* for 10 s again, discard supernatant, and resuspend the nanoparticles in 7 μL of 100% EtOH per bombardment planned. To resuspend the nanoparticles briefly sonicate using an ultrasonic water-bath sonicator. Usually two rounds of 5 s sonication.
8. Pipet 7 μL of DNA coated nanoparticles in the center of each macrocarrier and leave to dry. Spread across the center of macrocarrier surface with the use of a sterile pipette tip.

3.4.3 Bombardment

Biolistic bombardment is carried out with the Bio-Rad PDS-1000/He device. Rupture pressures of 900 pounds per square inch (PSI) must be used at a 9 cm distance between the stopping mesh and the sample. All work should be performed in a flow hood.

1. Load the rupture disk into the retaining cup using sterile tweezers.
2. Screw firmly the retaining cup with the rupture disk onto the gas acceleration tube at the top of the bombardment chamber.
3. Using sterile tweezers place the stopping screen into the macrocarrier launch assembly.
4. Screw the macrocarrier holder (with the plasmid DNA loaded macrocarrier) on top of the macrocarrier launch assembly.
5. Place the macrocarrier launch assembly into the bombardment chamber, second position from the top, and close the door.
6. Place the opened plate with the sporelings on the target shelf.
7. Press the vacuum button (second red button from the left) to "vac" position until vacuum reaches 27–28 in. Hg and immediately move button to "hold" position. Then keep the fire button pressed until rupture disk bursts (pressure reaches ~1000 PSI) and then release the fire button.
8. Release the vacuum.

9. Bombard each plate twice (we observed increased efficiency with the second bombardment).
10. Remove the plate from the chamber.
11. Unload macrocarrier launch assembly and rupture disk retaining cup.

3.4.4 Selection of Successful Transformants

1. After bombardment, incubate the plates, with the bottom side up again, for 2 days at 21 °C under continuous light with light intensity of 150 μmol/m^2/s.
2. Using a sterile scalpel transfer the spores on plates containing selection media.
3. Add 1–2 mL of sterile water on the plate and using a sterile spatula spread the spores on the entire surface of the plate.
4. Place the plates at 21 °C under continuous light with light intensity of 150 μmol/m^2/s for growth and selection.
5. After approximately a month untransformed sporelings will be completely bleached and successful transformants will be visible as bright green growths.
6. For constructs designed to express a fluorescent protein marker (such as the chloroplast codon optimized mTurquoise2 fluorescent protein, Fig. 5g–j) the first indication of successful chloroplast transformation can be observed 4 weeks after bombardment using a fluorescence stereo microscope.
7. Transfer successful transformants onto plates containing fresh selection media for another 4–6 weeks at 21 °C under continuous light with light intensity of 150 μmol/m^2/s.
8. When plants produce gemmae, transfer 5 gemmae (gemmae are clonal propagules produced from a single cell of the thallus) onto plates containing fresh selection media at 21 °C under continuous light with light intensity of 150 μmol/m^2/s.

3.5 Genotyping

Genotype 2-week-old gemmae (*see* **Note 7**) to check whether the plants are homoplastic:

1. Take small pieces (3 × 3 mm) of thalli from individual plants, place in a 1.5 mL Eppendorf tube and crush with an autoclaved micropestle in 100 μL genotyping buffer (*see* **Note 8**).
2. Place the tube(s) at 80 °C for 5 min.
3. Add 400 μL of sterile water to each tube.
4. Use 5 μL aliquot of the extract as a template for PCR using Phusion polymerase. PCR conditions: denaturation at 98 °C for 30 s; 35 cycles of: denaturation at 98 °C for 10 s, annealing at primer annealing temperature for 30 s, and extension at 72 °C for 15 s/kb; and final extension at 72 °C for 10 min. Check PCR products on a 1.5% (w/v) agarose gel (*see* **Note 9**).

4 Notes

1. pCkchlo, pCschlo and pUAP4 vectors, and L0 DNA parts are deposited on Addgene [9].
2. Chloroplast Loop assembly employs BsaI for Level 0 parts and is compatible with other Type IIS cloning systems. The adoption of SapI enzyme was based on the length of its recognition site (7 bp) which makes its occurrence less frequent in DNA sequences, minimizing the need for domestication. Domestication of internal BsaI and SapI sites is necessary when a DNA sequence of interest contains an internal BsaI or SapI recognition site. For domestication, overlapping PCR can be used. Briefly, the sequence can be amplified as two separate PCR fragments upstream (fragment 1) and downstream (fragment 2) the recognition site. The enzyme recognition site will form part of the reverse primer of fragment 1 and the forward primer of fragment 2, that will be specially designed with a single nucleotide mismatch to alter the recognition site (taking care to not alter amino acid composition if the region is protein coding). Amplification primers will also contain SapI recognition sites that will allow the two fragments to be ligated together into the pUAP4. For example if you need to domesticate the following sequence: 5′-atggcgtcaattagtggatgcgcagctgtgcctttgagc. . .. . .gcaacagcaaatggctattccagatt**GGTCTC** *agccaatgcaactgaagggc . . .. . .gattatcacagagggtgatggaaggactatatactaa*-3′

   (fragment 1: underlined sequence, BsaI recognition sequence: sequence in bold capital letters and fragment 2: italicized sequence), the primers to be designed are:

   Primer-fragment1-F: 5′-cga**GCTCTTC** gtctcgaatggcgtcaattagtggatgc-3′.

   Primer-fragment1-R: 5′-cta**GCTCTTC** gCgaccaatctggaatag-3′.

   Primer-fragment2-F: 5′-cga**GCTCTTC** gtcGcagccaatgcaactgaagggc-3′.

   Primer-fragment2-R: 5′-cta**GCTCTTC** gtctcaaagcttagtatatagtccttccatc-3′.

   (SapI recognition sequence: sequence in bold capital letters, common syntax overhangs: underlined sequence and primer mismatch: sequence in capital letter). Primer-fragment1-F and Primer-fragment2-F are designed according to Level 0 parts primer design.
3. The DNA concentration of donor plasmids, containing the parts to be assembled, and acceptor plasmids, into which the parts will be assembled, should be 15 and 7.5 nM respectively

[6]. To calculate donor plasmid concentration (ng/ μL), divide donor plasmid length by 100. For acceptor plasmid divide by 200. Then prepare 10 μL aliquots of each DNA part with the appropriate concentration.

For example, if donor "plasmid X" size is 4200 bp, you need to prepare a 10 μL aliquot with a concentration of 42 ng/ μL (4200/100). If the original concentration of the" plasmid X" miniprep is 100 ng/μL then you need to mix 4.2 μL of "plasmid X" sample with 5.8 μL of dH_2O. That will give the desired 10 μL aliquot with 42 ng/μL concentration.

4. For Loop assembly reactions, different buffer combination and/or enzymes from different companies can be used. For example, the NEB CutSmart buffer (#B7204S) supplemented with 1 mM final concentration ATP can replace the NEB T4 ligase buffer in the Odd Level master mix. Similarly, the NEB SapI enzyme (#R0569S) and the NEB CutSmart buffer supplemented with 1 mM final concentration ATP can replace the Thermo Fisher T4 ligase buffer in the Even Level master mix.
5. Milton sterilization solution should be prepared fresh every time. Milton mini sterilizing tablets are originally used to sterilize babies' feeding utensils.
6. Using water-free 100% EtOH is critical. Adding molecular sieves in the 100% EtOH will help keep water from entering the solvent.
7. If the plant does not produce gemmae after a prolonged period of selection (usually 2–3 months), a thallus fragment can be used for genotyping instead. Alternatively, plants can be moved on plates that contain a lower spectinomycin concentration of 100 μg/mL or no spectinomycin to facilitate gemmae production.
8. If there are issues with the quality of the "crude" DNA sample obtained using the genotyping buffer, a standard CTAB [14] genomic DNA extraction protocol can be used instead.
9. The transformation vector is comprised of two homologous regions that correspond to 1.5–2 kb flanking sequences upstream and downstream of the integration region. Genotyping primers should be designed to bind upstream of the 5′ end of the left homologous and downstream the 3′ end of the right homologous regions cloned into the transformation construct. If the plant is homoplastic the PCR amplified fragment should correspond approximately to the size of the transgene. If the line is not homoplastic an additional PCR fragment, that corresponds to the size of the native genomic sequence, will also be amplified.

Acknowledgments

We would like to thank Katrin Geisler for advice on the use of DNAdel™ nanoparticles and Linda Silvestri for assistance with plant growth and technical support.

References

1. Bowman JL, Kohchi T, Yamato KT, Jenkins J, Shu S, Ishizaki K, Yamaoka S, Nishihama R, Nakamura Y, Berger F, Adam C, Aki SS, Althoff F, Araki T, Arteaga-Vazquez MA, Balasubrmanian S, Barry K, Bauer D, Boehm CR, Briginshaw L, Caballero-Perez J, Catarino B, Chen F, Chiyoda S, Chovatia M, Davies KM, Delmans M, Demura T, Dierschke T, Dolan L, Dorantes-Acosta AE, Eklund DM, Florent SN, Flores-Sandoval E, Fujiyama A, Fukuzawa H, Galik B, Grimanelli D, Grimwood J, Grossniklaus U, Hamada T, Haseloff J, Hetherington AJ, Higo A, Hirakawa Y, Hundley HN, Ikeda Y, Inoue K, Inoue S-I, Ishida S, Jia Q, Kakita M, Kanazawa T, Kawai Y, Kawashima T, Kennedy M, Kinose K, Kinoshita T, Kohara Y, Koide E, Komatsu K, Kopischke S, Kubo M, Kyozuka J, Lagercrantz U, Lin S-S, Lindquist E, Lipzen AM, Lu C-W, De Luna E, Martienssen RA, Minamino N, Mizutani M, Mizutani M, Mochizuki N, Monte I, Mosher R, Nagasaki H, Nakagami H, Naramoto S, Nishitani K, Ohtani M, Okamoto T, Okumura M, Phillips J, Pollak B, Reinders A, Rövekamp M, Sano R, Sawa S, Schmid MW, Shirakawa M, Solano R, Spunde A, Suetsugu N, Sugano S, Sugiyama A, Sun R, Suzuki Y, Takenaka M, Takezawa D, Tomogane H, Tsuzuki M, Ueda T, Umeda M, Ward JM, Watanabe Y, Yazaki K, Yokoyama R, Yoshitake Y, Yotsui I, Zachgo S, Schmutz J (2017) Insights into land plant evolution garnered from the *Marchantia polymorpha* genome. Cell 171:287–304
2. Boehm CR, Ueda M, Nishimura Y, Shikanai T, Haseloff J (2016) A cyan fluorescent reporter expressed from the chloroplast genome of *Marchantia polymorpha*. Plant Cell Physiol 57:291–299
3. Chiyoda S, Linley PJ, Yamato KT, Fukuzawa H, Yokota A, Kohchi T (2007) Simple and efficient plastid transformation system for the liverwort *Marchantia polymorpha* L. suspension-culture cells. Transgenic Res 16:41–49
4. Engler C, Gruetzner R, Kandzia R, Marillonnet S (2009) Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PLoS One 4:e5553
5. Werner S, Engler C, Weber E, Gruetzner R, Marillonnet S (2012) Fast track assembly of multigene constructs using Golden Gate cloning and the MoClo system. Bioeng Bugs 3:38–43
6. Pollak B, Cerda A, Delmans M, Álamos S, Moyano T, West A, Gutiérrez RA, Patron NJ, Federici F, Haseloff J (2019) Loop assembly: a simple and open system for recursive fabrication of DNA circuits. New Phytol 222:628–640
7. Liu W, Stewart CN Jr (2015) Plant synthetic biology. Trends Plant Sci 20:309–317
8. Patron NJ, Orzaez D, Marillonnet S, Warzecha H, Matthewman C, Youles M, Raitskin O, Leveau A, Farré G, Rogers C, Smith A, Hibberd J, Webb AAR, Locke J, Schornack S, Ajioka J, Baulcombe DC, Zipfel C, Kamoun S, Jones JDG, Kuhn H, Robatzek S, Van Esse HP, Sanders D, Oldroyd G, Martin C, Field R, O'Connor S, Fox S, Wulff B, Miller B, Breakspear A, Radhakrishnan G, Delaux P-M, Loqué D, Granell A, Tissier A, Shih P, Brutnell TP, Quick WP, Rischer H, Fraser PD, Aharoni A, Raines C, South PF, Ané J-M, Hamberger BR, Langdale J, Stougaard J, Bouwmeester H, Udvardi M, Murray JAH, Ntoukakis V, Schäfer P, Denby K, Edwards KJ, Osbourn A, Haseloff J (2015) Standards for plant synthetic biology: a common syntax for exchange of DNA parts. New Phytol 208:13–19
9. Sauret-Güeto S, Frangedakis E, Silvestri L, Rebmann M, Tomaselli M, Markel K, Delmans M, West A, Patron NJ, Haseloff J (2020) Systematic tools for reprogramming plant gene expression in a simple model. ACS Synth Biol 9:864–882
10. Kahl L, Molloy J, Patron N, Matthewman C, Haseloff J, Grewal D, Johnson R, Endy D (2018) Opening options for material transfer. Nat Biotechnol 36:923–927
11. Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol 25:989–994

12. Ishizaki K, Nishihama R, Yamato KT, Kohchi T (2016) Molecular genetic tools and techniques for *Marchantia polymorpha* research. Plant Cell Physiol 57:262–270
13. Delmans M, Pollak B, Haseloff J (2017) MarpoDB: an open registry for *Marchantia polymorpha* genetic parts. Plant Cell Physiol 58:e5
14. Porebski S, Bailey LG, Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol Biol Rep 15(1):8–15