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Methods

Loop assembly: a simple and open system for recursive fabrication of DNA circuits

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Summary

- High-efficiency methods for DNA assembly have enabled the routine assembly of synthetic DNAs of increased size and complexity. However, these techniques require customization, elaborate vector sets or serial manipulations for the different stages of assembly.
- We have developed Loop assembly based on a recursive approach to DNA fabrication. The system makes use of two Type IIS restriction endonucleases and corresponding vector sets for efficient and parallel assembly of large DNA circuits. Standardized level 0 parts can be assembled into circuits containing 1, 4, 16 or more genes by looping between the two vector sets. The vectors also contain modular sites for hybrid assembly using sequence overlap methods.
- Loop assembly enables efficient and versatile DNA fabrication for plant transformation. We show the construction of plasmids up to 16 genes and 38 kb with high efficiency (> 80%). We have characterized Loop assembly on over 200 different DNA constructs and validated the fidelity of the method by high-throughput Illumina plasmid sequencing.
- Our method provides a simple generalized solution for DNA construction with standardized parts. The cloning system is provided under an OpenMTA license for unrestricted sharing and open access.

Introduction

Standardized approaches to the assembly of large DNAs have played an important role in the development of systematic strategies for the reprogramming of biological systems. This began with the implementation of idempotent assembly methods based on DNA digestion/ligation using standardized nested restriction endonuclease (RE) sites, such as the BioBrick assembly method (Knight, 2003; Shetty *et al.*, 2008). More recently, assembly techniques that enable the parallel assembly of multiple components in a single reaction have been established. These include methods that utilize long-sequence overlaps (Bitinaite *et al.*, 2007; Li & Elledge, 2007; Zhu *et al.*, 2007; Gibson *et al.*, 2009; Bryksin & Matsumura, 2010; Zhang *et al.*, 2012; Beyer *et al.*, 2015; Jin *et al.*, 2016), systems reliant on *in vivo* recombination (Ma *et al.*, 1987; Gibson *et al.*, 2008b; Joska *et al.*, 2014) and Golden Gate (Engler *et al.*, 2008)-based methods that rely on selective digestion and re-ligation of plasmid DNAs with Type IIS REs (Sarrion-Perdigones *et al.*, 2011, 2013; Weber *et al.*, 2011; Engler *et al.*, 2014; Storch *et al.*, 2015; Iverson *et al.*, 2016; Moore *et al.*, 2016). Type IIS and long-overlap-based methods have allowed increased scale and efficiency of DNA circuit assembly, whereas *in vivo* recombination remains the method of choice for genome-scale manipulations (Gibson *et al.*, 2008a,b, 2010a; Benders *et al.*, 2010; Karas *et al.*, 2012, 2013).

Gibson assembly, a sequence overlap-based method, was developed for the synthesis and assembly of *Mycoplasma* genomes (Gibson *et al.*, 2008a, 2010a) and enabled the assembly of DNAs of up to several hundred kilobases (kb) in one-pot isothermal reactions (Gibson *et al.*, 2009). This method has been widely adopted by the synthetic biology community, being scar-free, versatile and relatively efficient. However, Gibson assembly generally relies on the use of oligonucleotides to perform *in vitro* amplification of DNA fragments, which can be error prone (Keohavong & Thilly, 1989; Gibson *et al.*, 2010b; Potapov & Ong, 2017). The method is also sensitive to sequence composition and repeats, and hence efforts have been made to standardize and streamline Gibson assembly by including flanking unique

nucleotide sequences (UNSs) that can be used as long overlaps for the cloning of transcription units (TUs) into larger constructs (Torella *et al.*, 2013). Perhaps because of the flexible nature of Gibson assembly, a standard for composing elemental parts into TUs has not been proposed to date. Laboratories that employ Gibson assembly rely on their own set of rules and templates for DNA parts, and there has been no community-wide effort to develop a common standard.

By contrast, Type IIS assembly systems are virtually free of *ad hoc* design, and are highly efficient for both the assembly of TUs and the assembly of elementary parts into TUs (Patron, 2016). These methods do not require PCR amplification or fragment isolation, and allow the parallel assembly of a large number of DNA parts (Potapov *et al.*, 2018). Instead of PCR, these methods exploit Type IIS REs to generate fragments with short complementary overhangs that can be ligated in a one-pot reaction. Although this approach can be scarless, the application of standard overhangs (fusion sites) for DNA parts with a defined function (e.g. promoter, coding sequence (CDS), terminators) allows the same DNA parts to be re-assembled into multiple constructs without redesign or modification (Engler *et al.*, 2008; Sarrion-Perdigones *et al.*, 2011, 2013; Weber *et al.*, 2011; Lampropoulos *et al.*, 2013; Binder *et al.*, 2014). Recently, a common syntax has been proposed by developers and adopters of Type IIS cloning methods. This standard defines an unambiguous arrangement of 12 Type IIS overhangs that form boundaries between functional domains found within a generalized eukaryote gene (Patron *et al.*, 2015). The common syntax is based on the widely used MoClo and GoldenBraid standards, and has found acceptance in the plant field (Patron *et al.*, 2015) and iGEM in the form of PhytoBricks standard parts. The common syntax ensures that these Type IIS assembly systems can share a common stock of standardized DNA parts to be shared and used in an off-the-shelf manner. The establishment of a common standard for stock DNA parts also provides a prevailing syntax that enhances the transferability and reproducibility for the compilation of genetic instructions in different laboratories. The assembly of an exact copy of a genetic construct is possible simply by knowing its composition, eliminating unnecessary *ad hoc* design and enabling simple abstract descriptions that contain a precise implied sequence. However, Type IIS assembly systems require the refactoring or 'domestication' of DNA parts, generally performed through PCR or DNA synthesis. Domestication refers to the elimination of RE sites present in the DNA sequence before its use in the assembly system. To date, the most commonly used REs have been BsaI, BsmBI and BpiI, which have 6-bp recognition sites that, although not frequent on average, are regularly encountered in DNA sequences (Lin & O'Callaghan, 2018). Type IIS REs, such as SapI and AarI, with 7-bp recognition sites, can be used to lower the probability of finding sites requiring domestication, and are used in the Electra™ (ATUM, Newark, CA, USA) and GeneArt™ (ThermoFisher, Waltham, MA, USA) kits, respectively. Type IIS-based systems have found rapid acceptance in the synthetic biology field because of the need for robustness, scalability and compatibility with automated assembly methods. As synthetic biology is already at the point at which constructs can consist of multiple logic gates (Nielsen *et al.*, 2016), entire biosynthetic pathways (Temme *et al.*, 2012) or engineered genomic DNA (Richardson *et al.*, 2017), robust assembly methods, such as Type IIS assembly, are essential to enable the fabrication of higher order genetic constructs.

Despite much progress in the technical aspects of DNA construction and part reusability, restrictive intellectual property (IP) practices and material transfer agreements (MTAs) can hinder the sharing of DNA components in both the public and private sectors, delaying experimental work through paperwork and legal consultation. For this purpose, an international effort is underway to establish the OpenMTA (https://www.openmta.org) as a way of expediting the sharing of biological materials. The OpenMTA provides a legal template for free and unrestricted distribution of materials, providing a formal mechanism for effectively placing materials in the public domain, in a manner that extends existing practices. Open sharing of DNA assembly systems and parts through the OpenMTA will facilitate the engineering of new solutions for problems in human health, agriculture and the environment, such as those identified as Sustainable Development Goals by the United Nations (https://www.un.org/sustainabledevelopment) and Global Grand Challenges by the Gates Foundation (https://gcgh.grandchallenges.org).

Here, we present Loop assembly, a versatile, simple and efficient DNA fabrication system based on recursive DNA assembly. It combines all the benefits of Type IIS assembly, but requires only a set of eight plasmids to build constructs with theoretically unlimited length. As well as Type IIS assembly, the system integrates long-overlap assembly methods. In this way, four TUs can be assembled into multiple TUs using alternative methods, such as Gibson assembly via flanking UNSs (Torella *et al.*, 2013). In our method, Type IIS assemblies are performed through iterated 'loops'. Two sets of four plasmid vectors are provided, which allow alternating assembly cycles. First, Level 0 parts, defined by the PhytoBrick common syntax, are assembled into Level 1 TUs in each of four odd-numbered vectors using BsaI. Second, four Level 1 modules can then be assembled into a Level 2 construct in each of the four even-numbered vectors using SapI. Following this, Level 2 constructs can be combined by cloning back into odd-numbered vectors, using BsaI, to create Level 3 assemblies containing up to 16 TUs each. The iterative process of combining genetic modules, four at a time, can be continued without theoretical limit, alternating assembly steps between odd and even Loop vectors. As levels are used recursively, it is possible to create hybrid levels that can contain a mixture of parts from different levels of the same parity (i.e. Level 2 vectors combined with elements from Level 0 vectors). In addition, we have developed LoopDesigner, a software framework for *in silico* sequence handling and assembly design. The software tools are open source and available through *Github*, and Loop assembly vectors are provided through the OpenMTA for unrestricted use. We have developed and tested the Loop assembly system in different laboratories and provide data to support the efficiency and robustness of the method. We have assembled over 200 constructs with up to 16 TUs and over 38 kb in size. We have tested Loop constructs

in planta and validated their function in transgenic *Marchantia polymorpha*, and through transient expression in *Arabidopsis thaliana* protoplasts.

Materials and Methods

Construction of Loop assembly backbones

Loop assembly vectors were constructed using Gibson assembly (Gibson *et al.*, 2009). Several changes were made to a pGreenII vector (Hellens *et al.*, 2000) to obtain a basic plasmid backbone for the Loop assembly vectors: BsaI and SapI sites were removed from the plasmid using silent mutations when possible. In order to reduce issues with the stability of large constructs in bacteria (Moore *et al.*, 2016; Watson *et al.*, 2016), two nucleotides of the pGreenII ColEI-derived origin of replication were mutated, reversing it into the medium–low copy number pBR322 origin of replication. A region extending from the T-DNA left border to the hygromycin resistance gene cassette was replaced with the sequence of the pET15 vector (Haseloff, 1999) from the nptII nosT terminator to the UAS_{GAL4} promoter (bases 2851–3527). A spectinomycin resistance was cloned to replace the nptI cassette to provide a microbial selection marker for the pEven plasmids. UNSs were cloned into the kanamycin and spectinomycin version of the vector backbones after the 3′ end of the pET15 vector sequence and the right border. Finally, the Loop restriction enzyme sites (BsaI and SapI), overhangs and the lacZα cassette were cloned in between the UNSs, yielding the pOdd and pEven vectors. L0 plasmids used for Loop Type IIS assembly were assembled using Gibson assembly into a modified pUDP2 (BBa_P10500) plasmid, which contained a 20-bp random sequence (5′-TAGCCGGTCGAGTGATACA CTGAAGTCTC-3′) downstream of the 3′ convergent BsaI site and upstream of the BioBrick suffix, to provide nonhomologous flanking regions for correct orientation during overlap assembly.

DNA spacers

Random DNA sequences were retrieved from Random DNA Sequence Generator (https://www.faculty.ucr.edu/~mmaduro/random.htm), ordered as dsDNA fragments from IDT and assembled using Gibson assembly.

Plasmids and construct design

L0 parts used for DNA construction are described in Supporting Information Table S1; their sequences are included in the Supporting Information and are available through Addgene. Sequences for Loop plasmids and resulting multigene assemblies are included in Supporting Information.

The design of the constructs was performed using LoopDesigner software, installed on a local machine. The software was configured to use Loop assembly backbones together with BsaI and SapI REs, as well as A–B and α–ω overhangs. In addition, the definitions of 12 L0 part types were added to the software, based on the overhangs specified by the common syntax. The sequences of the L0 parts were added to the LoopDesigner database, assigning one of the defined part types, and assembled consequently into Level 1 and Level 2 constructs *in silico*. The concentrations of L0 parts and Level 1 constructs were adjusted to those suggested by LoopDesigner for 10-μl reactions.

Loop Type IIS assembly protocol

The Loop Type IIS assembly protocol was adapted from Patron (2016), and can be found at https://www.protocols.io/view/loop-assembly-pyqdpvw. An aliquot of 15 fmol of each part to be assembled was mixed with 7.5 fmol of the receiver plasmid in a final volume of 5 μl with distilled H_2O (dH_2O) (Table S2). The reaction mix, containing 3 μl of dH_2O, 1 μl of T4 DNA ligase buffer 10× (no. B0202; NEB, Ipswich, MA, USA), 0.5 μl of 1 mg ml^{-1} purified bovine serum albumin (1 : 20 dilution in dH_2O of BSA, Molecular Biology Grade 20 mg ml^{-1}, NEB cat. B9000), 0.25 μl of T4 DNA ligase at 400 U μl^{-1} (NEB cat. M0202) and 0.25 μl of corresponding restriction enzyme at 10 U μl^{-1} (BsaI NEB cat. R0535 or SapI NEB cat. R0569), was prepared on ice. Then, 5 μl of the reaction mix was combined with 5 μl of DNA mix for a reaction volume of 10 μl (Table S3) by pipetting, and incubated in a thermocycler using the program described in Table S4. For SapI reactions, T4 DNA ligase buffer was replaced by CutSmart buffer (NEB cat. B7204S) supplemented with 1 mM ATP; 1 μl of the reaction mix was added to 50 μl of chemically competent TOP10 cells (no. C4040100; ThermoFisher) and, following incubation at 42°C for 30 s, samples were left on ice for 5 min, 250 μl of Super Optimal broth with Catabolite repression (SOC) medium was added and cells were incubated at 37°C for 1 h. Finally, 5 μl of 25 mg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (no. B4252; Sigma-Aldrich), dissolved in dimethylsulfoxide (DMSO), was added and the cells were plated onto selective Lysogeny broth (LB)-agar plates supplemented with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (no. I6758; Sigma-Aldrich). Assembly reactions were also automated. The assembly reactions were identical, but scaled down to a total volume of 1 μl. Reactions were set up on a Labcyte Echo (San Jose, CA, USA) in 384-well plates and incubated on a thermal cycling machine using the same conditions as described above. Reactions were transformed into 4 μl competent XL10-Gold® Ultracompetent Cells (Agilent Technologies, Santa Clara, CA, USA) and plated onto eight-well selective LB-agar plates. Colonies were picked for growth in 1 ml of medium in 96-well plates on a Hamilton STARplus® platform (Reno, NV, USA).

Standardized PCR of transcriptional units

PCR using UNS oligonucleotides was performed at an annealing temperature of 60°C, with 35 cycles using Phusion High-Fidelity DNA polymerase (no. F-530; ThermoFisher) in 50-μl reactions, according to the manufacturer's instructions. Template was added to a final concentration of 20 pg μl^{-1}. DNA fragments were visualized using SYBR Safe DNA Gel Stain (no.

S33102; ThermoFisher) on a blue LED transilluminator (IORodeo, Pasadena, CA, USA). DNA purification was performed using a NucleoSpin Gel and PCR Clean-up purification kit (no. 740609.250; Macherey-Nagel, Düren, Germany). UNS primers used in TU amplification are listed in Table S5.

Validation by sequencing

The sequences of the assembled plasmids were verified by complete sequencing using 150-base pair paired-end reads on an Illumina MiSeq platform, and can be found in the EMBL-ENA database grouped under study PRJEB29863. Libraries were prepared using the Nextera XT DNA Library Prep Kit (no. FC-131-1096; Illumina Inc., San Diego, CA, USA), using the manufacturer's protocol modified to a one in four dilution. Reads were filtered and trimmed for low-quality bases and mapped to plasmids using the 'map to reference tool' from Geneious 8.1.8 software (https://www.geneious.com; Kearse *et al.*, 2012), with standard parameters. Sequence fidelity was determined manually.

Agrobacterium-mediated *Marchantia* transformation

Agrobacterium-mediated transformation was carried out as described previously (Ishizaki *et al.*, 2008), with the following exceptions: half of an archegonia-bearing sporangium (spore-head) was used for each transformation. Dried spore-heads were crushed in a 50-ml Falcon tube with a 15-ml Falcon tube and resuspended in 1 ml of water per spore-head. Resuspended spores were filtered through a 40-μm mesh (no. 352340; Corning Inc., NY, USA) and 1 ml of suspension was aliquoted into a 1.5-ml Eppendorf tube and centrifuged at 13 000 ***g*** for 1 min at room temperature. The supernatant was discarded and spores were resuspended in 1 ml of sterilization solution, and incubated at room temperature for 20 min at 150 rpm on an orbital shaker. The sterilization solution was prepared by dissolving one Milton mini-sterilizing tablet (Milton Pharmaceutical UK, Cheltenham, UK active ingredient, sodium dichloroisocyanurate CAS: 2893-78-9: 19.5% w/w) in 25 ml of sterile water. Samples were centrifuged at 13 000 ***g*** for 1 min, washed once with sterile water and resuspended in 100 μl of sterile water per spore-head used. One hundred microlitres of sterilized spores were inoculated onto half-strength Gamborg's B5 1% (w/v) agar plates and grown under constant fluorescent lighting (50–60 mol photons $m^{-2}s^{-1}$) upside down for 5 d until co-cultivation. Sporelings were co-cultivated with previously transformed and induced *Agrobacterium* GV2260 transformed with the pSoup plasmid (Hellens *et al.*, 2000) in 250-ml flasks containing 25 ml of half-strength Gamborg's B5 medium supplemented with 5% (w/v) sucrose, 0.1% (w/v) N-Z Amine A (Sigma cat. C7290), 0.03% (w/v) L-glutamine (Sigma cat. G8540) and 100 μM acetosyringone (Sigma-Aldrich cat. D134406) for 36 h, until washing and plating onto selective medium.

Laser scanning confocal microscopy

A microscope slide was fitted with a 65-μl Gene Frame (ThermoFisher cat. AB0577) and 65 μl of dH_2O was placed in the centre. *Marchantia gemmae* was carefully deposited on the drop of dH_2O using a small inoculation loop and a #0 coverslip was attached to the Gene Frame. Slides were examined on a Leica, Wetzlar, Germany TCS SP8 confocal microscope platform equipped with a white-light laser (WLL) device. Imaging was conducted using a Leica HC PL APO 20× CS2 air objective with a sequential scanning mode with laser wavelengths of 405, 488 and 515 nm, capturing emitted fluorescence at 450–482-, 492–512- and 520–550-nm windows, respectively, in each sequential scan. Z-stacks were collected every 5 μm for the complete volume range and maximum intensity projections were processed using ImageJ software. Fluorescence bleedthrough from the blue pseudocoloured channel (membrane-localized enhanced green fluorescent protein (eGFP)) into the green pseudocoloured channel (nuclear-localized Venus) was eliminated using custom *Python* scripts which subtracted 20% of the value of pixels present in the blue channel to the green channel. Images were edited to scale the pixel intensity to the full 8-bit range and a merged image was processed.

Transient expression in Arabidopsis mesophyll protoplasts

Well-expanded leaves from 3–4-wk-old Arabidopsis plants (Columbia-0) were used for protoplast transfection. Plants were grown at 22°C in low-light (75 μmol $m^{-2}s^{-1}$) and short-photoperiod (12 h : 12 h, light : dark) conditions. Protoplasts were isolated and polyethylene glycol (PEG) transfected according to Yoo *et al.* (2007). For transfection, 6 μl of Loop L2 plasmids (2 μg μl^{-1}), isolated by a NucleoBond Xtra Midi/Maxi purification kit (Macherey-Nagel cat. 740410.50), were used. Transfected protoplasts were incubated for 12 h in light and then visualized by epifluorescent microscopy in a Neubauer chamber (Hirschmann Laborgeräte, Eberstadt, Germany).

Epifluorescence microscopy

Transfected protoplasts were visualized using a Nikon Ni microscope (Minato, Tokyo, Japan) equipped with 49021 ET – EBFP2/Coumarin/Attenuated DAPI (excitation, 405/20 nm; dichroic, 425 nm; emission, 460/50 nm), 96227 AT-EYFP (excitation, 495/20 nm; dichroic, 515 nm; emission, 540/30 nm), 96223 AT-ECFP/C (excitation, 495/20 nm; dichroic, 515 nm; emission, 540/30 nm) and 96312 G-2E/C (excitation, 540/20 nm; dichroic, 565 nm; emission, 620/60 nm) filter cubes.

LoopDesigner

In order to implement an object-oriented model for Loop assembly, we built a PartsDB library (https://github.com/HaseloffLab/PartsDB) to define several interlinked classes, each of which is associated with a table in a relational SQL database. The structure of LoopDesigner is built around a *Part* class, which either represents an ordered collection of children parts from which it is assembled, or a DNA sequence in the case of L0 parts. In this way, we ensured that the actual DNA sequence is only stored once, and the sequences of L1 and higher parts are constructed

on demand from the relational links. In addition, each *Part* is associated with one of the *Backbone* instances which, together with a *Part* sequence, represents a complete Loop assembly plasmid. Every instance of a *Backbone* class is a combination of a *Base Sequence* and a donor *Restriction Enzyme Site*, for example, pOdd 1-4 and pEven 1-4 are *Backbone* instances in the schema described in this article. *Base Sequence* represents a type of receiver plasmid, for example, pOdd and pEven, and is composed of a DNA sequence of the plasmid and an instance of a receiver *Restriction Enzyme Site*. Finally, *Restriction Enzyme Site* class is composed of a *Restriction Enzyme* instance, which stores the restriction enzyme recognition sequence, and a pair of overhang sequences, which can be either receiver or donor overhangs.

Results

Loop assembly

The Loop assembly kit consists of two sets of plasmids that participate in a cyclic assembly process. Type IIS REs, BsaI and SapI, are used alternately for the recursive assembly of genetic modules into a quartet of either odd (L1, L3,. . .) or even (L2, L4,. . .) receiver plasmids. At each step in the assembly 'loop', four genetic modules are combined into a receiver plasmid (Fig. 1a). Odd and even level plasmids use alternating types of antibiotic selection, kanamycin resistance for odd levels (pOdd plasmids) and spectinomycin resistance for even levels (pEven plasmids), to enable the use of a one-pot digestion–ligation assembly reaction (Engler *et al.*, 2008). At each level (except for TU assembly from L0 parts), four parental plasmids are required, leading to an exponential increase in the number of TUs by a factor of four per level (Fig. 1b).

Plasmids in Loop assembly act as both donors and receivers because of the special arrangement of the RE sites. The odd receiver plasmids contain a pair of divergent BsaI sites that are removed in the cloning reaction, whereas a pair of convergent SapI sites, flanking the BsaI sites, allows the odd plasmids to act as donors for assembly into the following level. Similarly, the even plasmids contain a pair of divergent SapI sites flanked by convergent BsaI sites (Fig. 2a). On digestion, donor plasmids release DNA fragments (between the convergent RE sites) with specific overhangs that define the direction and position in the assembly, whereas the receiver plasmids release the divergent RE sites allowing for the assembly of the donor fragments.

The overhangs created by the BsaI digestion of the odd receivers allow the construction of TUs from any parts that are compatible with the PhytoBrick standard (Patron *et al.*, 2015), such as L0 parts derived from MoClo and GoldenBraid plasmid libraries (if free of SapI sites). BsaI overhang sequences are termed A, B, C, E and F, with A and F designated as flanking terminal overhangs, and SapI overhang sequences are termed α, β, γ, ε and ω, with α and ω designated as flanking terminal overhangs. Examples of odd and even level assemblies are shown in (Fig. 2b,c).

Each reaction requires four donor plasmids (or DNA spacers) for successful assembly into a receiver of the next level. In order to provide a replacement for assemblies with less than four fragments, we designed 200-bp-long 'universal spacer' parts comprising random DNA sequence free of BsaI and SapI sites. Plasmids containing spacers with flanking terminal overhangs are provided for odd (pOdd-spacer) and even (pEven-spacer) levels. They can be used for direct assembly into any of the four receiver plasmids of their corresponding level (Fig. S1).

Assembly of synthetic promoters

The recursive nature of Loop assembly allows one to mix parts from different levels, but with the same parity. For example, a multimeric promoter might be constructed from elemental parts through recursive assembly. Figure 3 shows the generation of synthetic promoters by cloning L0 functional domains (e.g. transcription factor (TF) recognition sites and minimum promoter sequences) with flanking terminal overhangs into specific L1 plasmid positions, which determine the order of motif arrangement in the following L2 assembly. Different TF recognition sites can be used in positions 1 (α and β overhangs), 2 (β and γ overhangs) and 3 (γ and ε overhangs), whereas a minimal promoter sequence is placed in position 4 (ε and ω overhangs) of L1 receiver plasmids. These elements can then be composed in specific order. In this example, different combinations of TF binding sites and minimal promoter were cloned into positions 1 (A and B overhangs) and 2 (B and C overhangs) of L2 receiver plasmids. The resulting composite promoter elements could be mixed with standard L0 gene parts, to create a customized hybrid gene assembly in an odd level plasmid (Fig. 3a).

Using this approach, we assembled three fluorescent reporters with synthetic promoters comprising multimeric binding sites. The promoters included binding domains for the yeast TFs GAL4 (Guarente *et al.*, 1982; West *et al.*, 1984; Giniger *et al.*, 1985) and HAP1 (Zhang & Guarente, 1994), a cytokinin (CK) operator (Müller & Sheen, 2008) and a minimal cauliflower mosaic virus (CaMV) 35S promoter (Benfey & Chua, 1990) derivative (F. Federici and J. Haseloff, unpublished results) driving a Venus fluorescent protein (Nagai *et al.*, 2002). The resulting reporters were composed of the same elements, but with differing motif arrangements, containing 13 nucleotide scars between the motifs. Each reporter contained three dimeric binding domains for GAL4, three dimeric binding domains for HAP1, one dimeric CK operator binding domain and the minimal CaMV 35S promoter (see Notes S1). The composite synthetic promoters, which were the result of 20 different assembly reactions, were verified through sequencing and showed no sequence errors. The sequences of the final constructs (pL3-1_PC1, pL3-1_PC2 and pL3-1_PC3) can be found in the Supporting Information.

The recursive nature of Loop assembly also enables hybrid assemblies of multiple TUs derived from donor plasmids from different levels (i.e. three Level 1 and one Level 3 plasmids). These can be assembled into a hybrid even receiver plasmid, providing further flexibility in the fabrication of genetic constructs (Fig. 3b).

UNSs for standardized overlap assembly

Apart from their capacity for Type IIS assembly, Loop vectors were designed for long-overlap assembly techniques. Loop

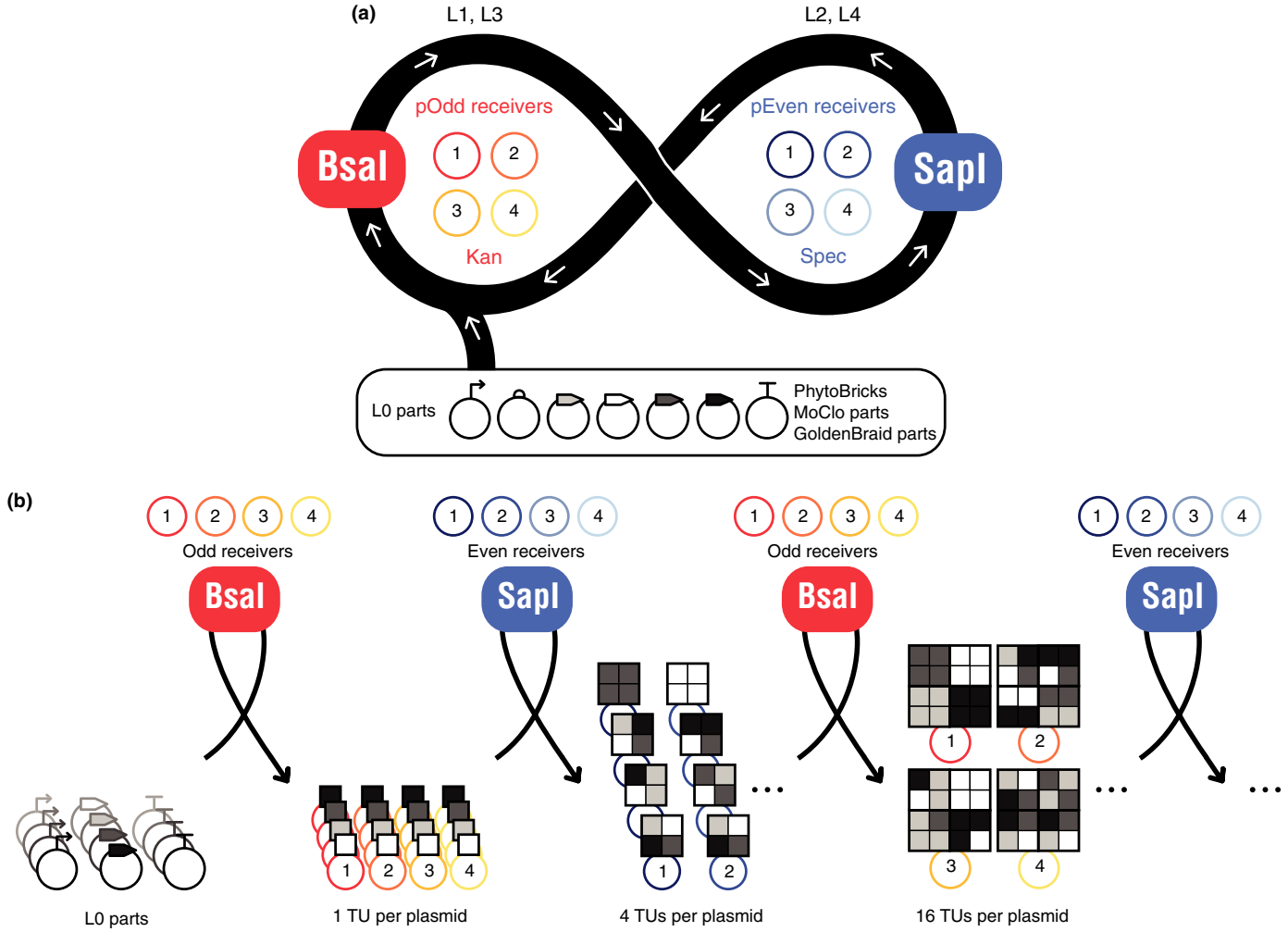

Fig. 1 Overview of Loop assembly. (a) Loop assembly workflow. L0 parts are assembled to L1 transcription units (TUs) into one pOdd receiver by BsaI-mediated Type IIS assembly. L1 TUs are assembled to L2 multi-TUs into one pEven receiver by SapI-mediated Type IIS assembly. This workflow is then repeated for higher level assemblies. Only four odd level and four even level receiver plasmids are required for Loop assembly. (b) Combinatorial and exponential assembly. L0 parts can be assembled to L1 TUs into any of the four positions of odd receivers. Genetic modules can easily be swapped in each TU arrangement and receiver position. L1 TUs can then be assembled into L2 multi-TUs with variable combinations of the L1 TUs, also into any of the four positions of the even receivers. Each round of assembly generates four assembled plasmids, and consequent rounds of assembly increase the number of TUs by a factor of four, leading to an exponential increase in TU number.

plasmids contain UNSs that allow the use of standard primers for the amplification of TUs derived from Type IIS DNA parts (PhytoBricks, MoClo and GoldenBraid), as these can be assembled into UNS-flanked TUs by BsaI-mediated Type IIS assembly. Alternatively, TUs can be assembled from PCR fragments or DNA synthesis into Loop plasmids by overlap assembly methods, such as Gibson assembly (Fig. 4a). Each Loop plasmid contains two flanking UNSs and a terminal UNS_x. TUs can be assembled into a multi-TU destination plasmid (pUNSDest) using overlap assembly methods (Fig. 4b). UNSs have been designed following a number of guidelines to provide enhanced performance in PCRs and overlap assembly. Design rules are listed in Methods S1 and sequences are provided in Table S6. Forward and reverse standard primers correspond to the first 20 bp of each UNS in both forward and reverse complement orientations, respectively, and are provided in Table S5. UNSs have the advantage that they are designed for highly efficient PCR with standard conditions (60°C, 35 cycles), resulting in single amplicons with high yields (Fig. S2). This eliminates the need for gel purification during the workflow of Gibson assembly, if appropriate on-column purification is performed.

Reliability of Loop assembly

To evaluate the reliability of the technique, we tested L1 Type IIS Loop assemblies in different laboratories, and obtained consistent results (Table 1). We assembled over 200 plasmids using the Type IIS pathway for Levels 1–3 and obtained average assembly efficiency between 83% and 97%, depending on the level of assembly and complexity of the constructs (Table 1; Notes S2). This was evaluated though DNA profiling by means of RE digestion (Fig. S3). Further, we performed Illumina sequencing of 92

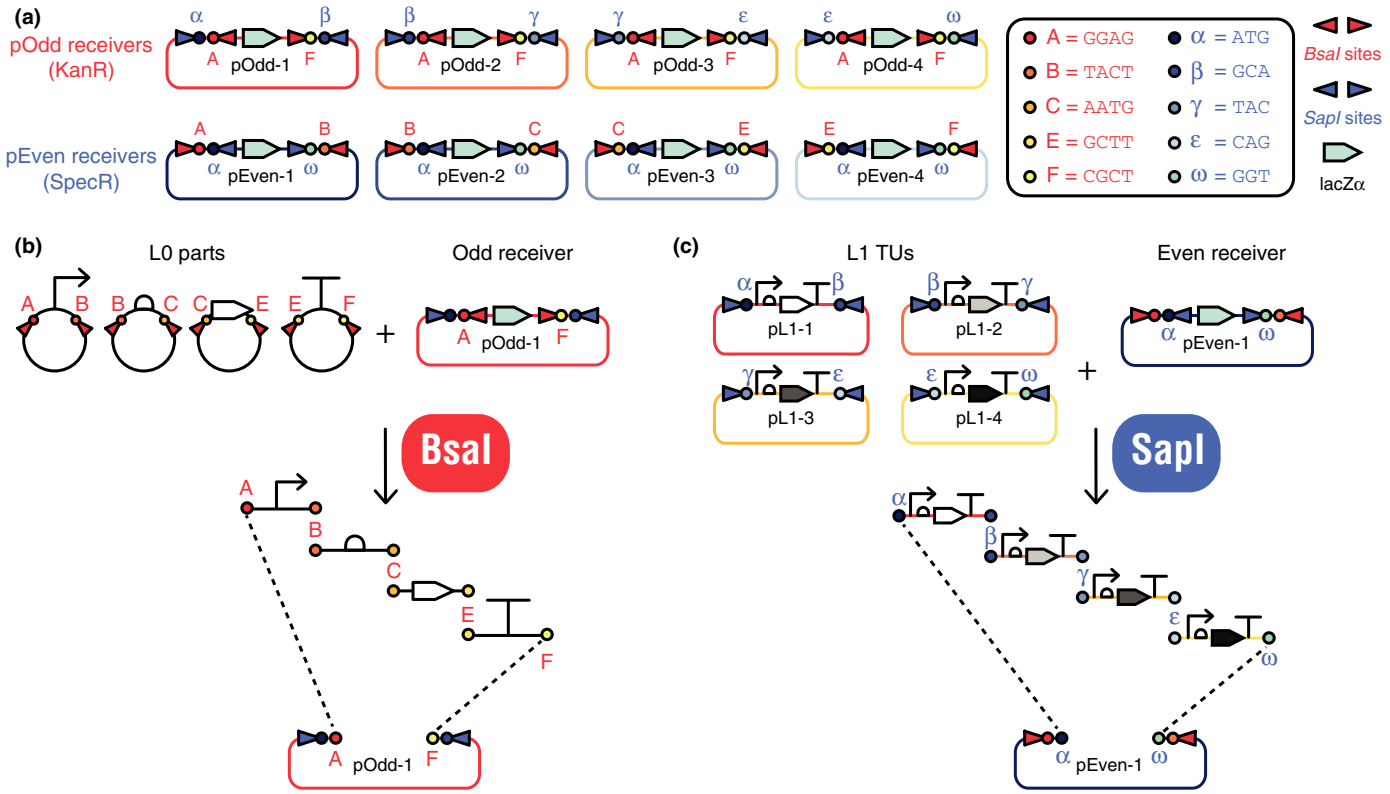

Fig. 2 Loop assembly schema. (a) Loop receiver plasmids. Each of the four pOdd and pEven receiver plasmids has a specific set of SapI (3 bp) and BsaI (4 bp) convergent overhangs, respectively, required for higher level assembly. Odd receivers contain diverging BsaI restriction sites and terminal overhangs according to the common syntax, making them compatible for cloning L0 parts into pOdd plasmids. They contain SapI converging sites with donor overhangs for directing SapI-mediated Type IIS assembly into even level receivers. pEven plasmids have SapI diverging restriction sites and terminal overhangs to receive parts from pOdd plasmids. For higher level assemblies, pEven plasmids contain converging BsaI sites with donor overhangs for BsaI-mediated Type IIS assembly into pOdd plasmids. (b) Loop odd level assembly. L0 DNA parts containing overhangs defined in the common syntax are assembled into a Loop odd level receiver. BsaI digestion releases the DNA modules, which are assembled into an even level receiver by directional assembly defined by 4-bp overhangs. pOdd plasmids contain A and F overhangs as terminal overhangs for receiving parts, which are flanked by convergent SapI restriction sites with 3-bp donor overhangs for further assembly. (c) Loop even level assembly. Four previously assembled pL1 transcription units (TUs) are assembled into a pEven plasmid. SapI digestion releases TUs from pL1 plasmids, which are assembled into an even level receiver by directional assembly defined by 3-bp overhangs. pEven plasmids contain α and ω overhangs as terminal overhangs, which are flanked by convergent BsaI restriction sites with donor overhangs defined in the common syntax required for further assembly.

Level 2 and Level 3 assembled constructs to validate Loop assembly fidelity at the sequence level, to determine whether the reaction had produced correct assemblies and whether mutations had been introduced by our method. We found that 95.4% of constructs assembled correctly with 98.8% of overhang scars present at the expected junctions. Overall, 99.8% of nucleotides were correctly assembled, and the few incorrect constructs showed missing regions as a result of misassembly, rather than sequence errors *per se* (Table S7).

In planta activity of Loop plasmids

Loop vectors were derived from the pGreenII (Hellens *et al.*, 2000) plant binary transformation vector, but decoupled from plant selection markers (see the Materials and Methods section), to enable their introduction during assembly. As in pGreenII, Loop plasmids contain elements for propagation in *Agrobacterium tumefaciens* and are capable of *Agrobacterium*-mediated plant transformation. We have tested the application of Loop constructs in plant developmental biology by assembling TUs composed of fluorescent proteins, localization tags and endogenous promoters. This allowed us to highlight cellular features and track patterns of gene expression *in planta*. A Level 2 construct (pL2-1_TPL) containing four TUs composed of a HygR selectable marker, an mTurquoise2-N7 nuclear-localized reporter driven by an Mp*EF1*α constitutive promoter (Nagaya *et al.*, 2011; Althoff *et al.*, 2014), a Venus-N7 nuclear-localized reporter driven by an Mp*TPL* tissue-specific promoter (Flores-Sandoval *et al.*, 2015) and an eGFP-Lti6b membrane-localized marker driven by an Mp*EF1*α promoter was assembled from L0 parts (Table S1) using Loop assembly, and transformed into *Marchantia polymorpha* (Marchantia). Regenerated transformants were obtained and clonal propagules called *gemmae* were examined using confocal microscopy. All three fluorescent protein reporter genes were expressed and allowed the visualization of distinct cellular and subcellular features across the tissue (Fig. 5).

In addition, four L1 TUs that had been constructed by Type IIS Loop assembly were assembled into a multi-TU destination

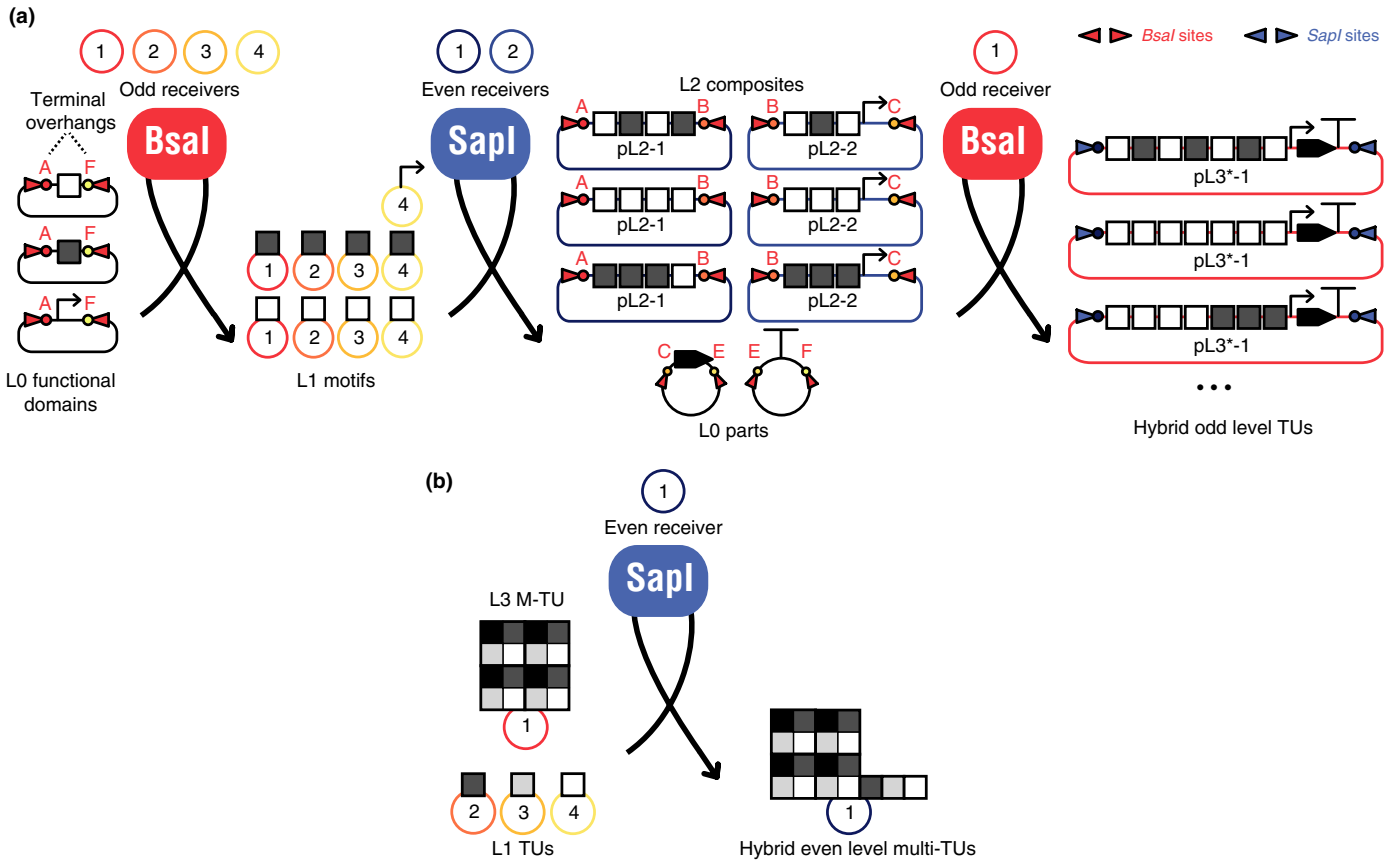

Fig. 3 Hybrid assembly. (a) Synthetic promoter assembly. L0 functional domains flanked by terminal overhangs are assembled into odd level receivers in any given position. L1 motifs are then assembled into L2 composites with differing arrangements into positions 1 and 2. L2 composites in positions 1 and 2 are used in a hybrid assembly with L0 parts to generate a hybrid odd level transcription unit (TU) with a synthetic promoter composed of the L0 functional domains in the defined arrangement. (b) Mixed level assembly. L3 and L1 parts are assembled into an even level receiver generating a hybrid even level multi-TU plasmid.

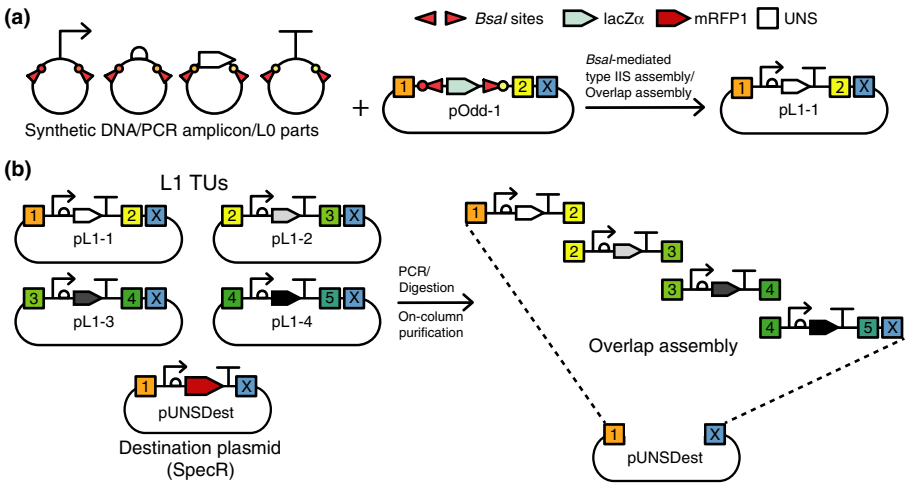

Fig. 4 Loop overlap assembly. (a) Transcription unit (TU) assembly for overlap assembly. Unique nucleotide sequence (UNS)-flanked transcription units (TUs) can be generated by either standard L0 BsaI-mediated Type IIS assembly or overlap assembly methods using PCR fragments or DNA synthesis. TUs produced by overlap assembly are only compatible with the overlap assembly pathway, but do not require domestication. (b) Standardized overlap assembly. Linear UNS-flanked TUs are amplified by PCR or excised from plasmids by digestion by uncommon restriction enzymes. Linear UNS-flanked TUs are then assembled to the destination plasmid pUNSDest by overlap assembly methods.

plasmid using Gibson assembly. Transfected protoplasts showed the expression of the engineered fluorescent reporters in their expected localizations (Fig. S4), providing a fast and efficient system to evaluate the functionality of Loop constructs. Plasmid maps for the constructs are provided in Fig. S5.

Loop assembly design automation

We have developed software tools to aid Loop assembly experiments. We developed LoopDesigner, a web application that facilitates: (1) the sequence design and domestication of Level 0

Table 1 Loop assembly efficiency.

Level	Constructs (no.)	TU (no.)	Average length (bp)	Overall efficiency* (%)	Average efficiency† (%)
Lab 1					
L1	104	1	6243	96	97
L2	79	4	13 519	82	88
L3	23	16	26 731	81	83
Hybrid	3	Var.	5473	100	100
Lab 2					
L1	14	1	5570	91	91
UNS overlap	5	4	12 548	71	71

*Overall efficiency calculated as the total number of samples with correct restriction digest (RD) patterns over the total number of samples tested.
†Average efficiency calculated as the mean of correct RD patterns over the number of samples tested per construct.
TU, transcription unit; UNS, unique nucleotide sequence.

DNA parts; (2) the generation of a Loop assembly parts database; and (3) the simulation of Loop assembly reactions and the resulting plasmid maps and sequences (Fig. 6). Input L0 sequences are domesticated by identifying unwanted RE sites and removing them by the introduction of synonymous mutations. Appropriate BsaI overhangs are added according to the rules of the common syntax for DNA parts and stored in the parts database. (See Materials and Methods for a detailed description of the LoopDesigner implementation.) We invite readers to visit the LoopDesigner web tool available at loopdesigner.herokuapp.com (supported in Google Chrome) for exploring Loop assembly techniques. The source code of LoopDesigner is available at *GitHub* (https://github.com/HaseloffLab/LoopDB LoopDesigner branch), and provided under a Massachusetts Institute of Technology (MIT) license.

Discussion

The design of Loop assembly was inspired by existing assembly methods, such as GoldenBraid, MoClo and standardized Gibson assembly. We attempted to integrate these techniques into a general-purpose DNA assembly system. Loop assembly combines the recursive use of two restriction enzymes and plasmid sets which, together, create a simple and versatile Type IIS assembly platform. Type IIS RE sites are employed in head-to-head configurations, eliminating the requirement for end-linkers used in MoClo systems. Instead, restriction sites for successive levels are integrated in receiver plasmids, as in GoldenBraid, but using quaternary assembly parity instead of binary. This enables the faster assembly of large constructs, but demands all four positions to be filled by either TUs or by spacers. Fixing the number of donor parts allows systematization without increasing the complexity of assembly, with standardized reactions containing a determined number of DNA parts and overhangs. Further, the recursive nature of Loop assembly enables the usage of a compact plasmid library, whilst providing versatile construction strategies. We show the use of recursive assembly for the fabrication of complex DNA, such as synthetic promoters composed of repetitive sequences and hybrid levels. Type IIS restriction sites in the Loop vectors are flanked by standardized UNSs, enabling the use of Loop vectors with overlap assembly methods and the reuse of oligonucleotides for assembly. This provides users of Gibson and overlap assembly methods with the capacity to tap into libraries of domesticated DNA parts already available. We have demonstrated the high efficiency of Loop assembly by generating a variety of constructs with different numbers of TUs, achieving reliable assembly of constructs up to 16 TUs composed of 56 individual parts. In addition, we have used Loop assembly for the generation of multispectral reporter constructs and have shown their activity *in planta*.

The use and characterization of the products of Loop assembly demonstrates that it is a robust and reliable DNA assembly system regardless of the levels and types of parts. Loop assemblies varying in size, total number of fragments and DNA composition were performed in order to provide an accurate estimate of the method performance in routine use. The high rate of successful assemblies, even in the absence of cPCR pre-selection, considerably decreases the effort and time required for DNA construction. Further, the system takes advantage of (1) a common syntax for DNA parts, (2) a simple, recursive assembly scheme, (3) a small set of plasmid vectors and (4) streamlined protocols, to provide a streamlined and logical framework for assembly that will enable rapid adoption by students and nonspecialists. As Loop assembly integrates Type IIS and overlap assembly, it encourages the development of a community around a DNA construction system, yielding a growing collection of DNA parts and composites. The wide compatibility of Loop assembly facilitates proper curation and improvement of DNA part collections through collaboration, easier exchange and transfer of genetic modules between laboratories, and cross-validation. The ability to use either overlap or Type IIS assembly provides further flexibility in making DNA constructions in which sequence alterations introduced by the removal of illegal RE sites are not desirable (such as for experiments involving native genetic sequences), or when the assembly fails by one of the pathways.

Although the falling costs of DNA synthesis suggest that the DNA synthesis of transcriptional units or even chromosomes might eventually be time and cost-effective, synthetic biology requires the capacity for rapid, high-throughput and combinatorial assemblies. This is necessary for the characterization and troubleshooting of smaller DNA parts and circuits before compiling high-level devices and systems. Assembly systems that are tailored to exploit the opportunities provided by automation technologies will undoubtedly benefit from robotics platforms. Automated design and liquid handling platforms for the fabrication of DNA constructs have already been adopted by some, and the technologies are rapidly expanding: at the high end of the market, platforms such as the Echo (Labcyte) are enabling miniaturization and increasing throughput, yielding a substantial reduction in reaction costs (Kanigowska *et al.*, 2016), whereas low-cost platforms, such as the OT-One S (OpenTrons), are aiming to make automated pipetting affordable in every laboratory.

To enable the facile design of constructs, we have developed LoopDesigner, a software framework that provides an interface

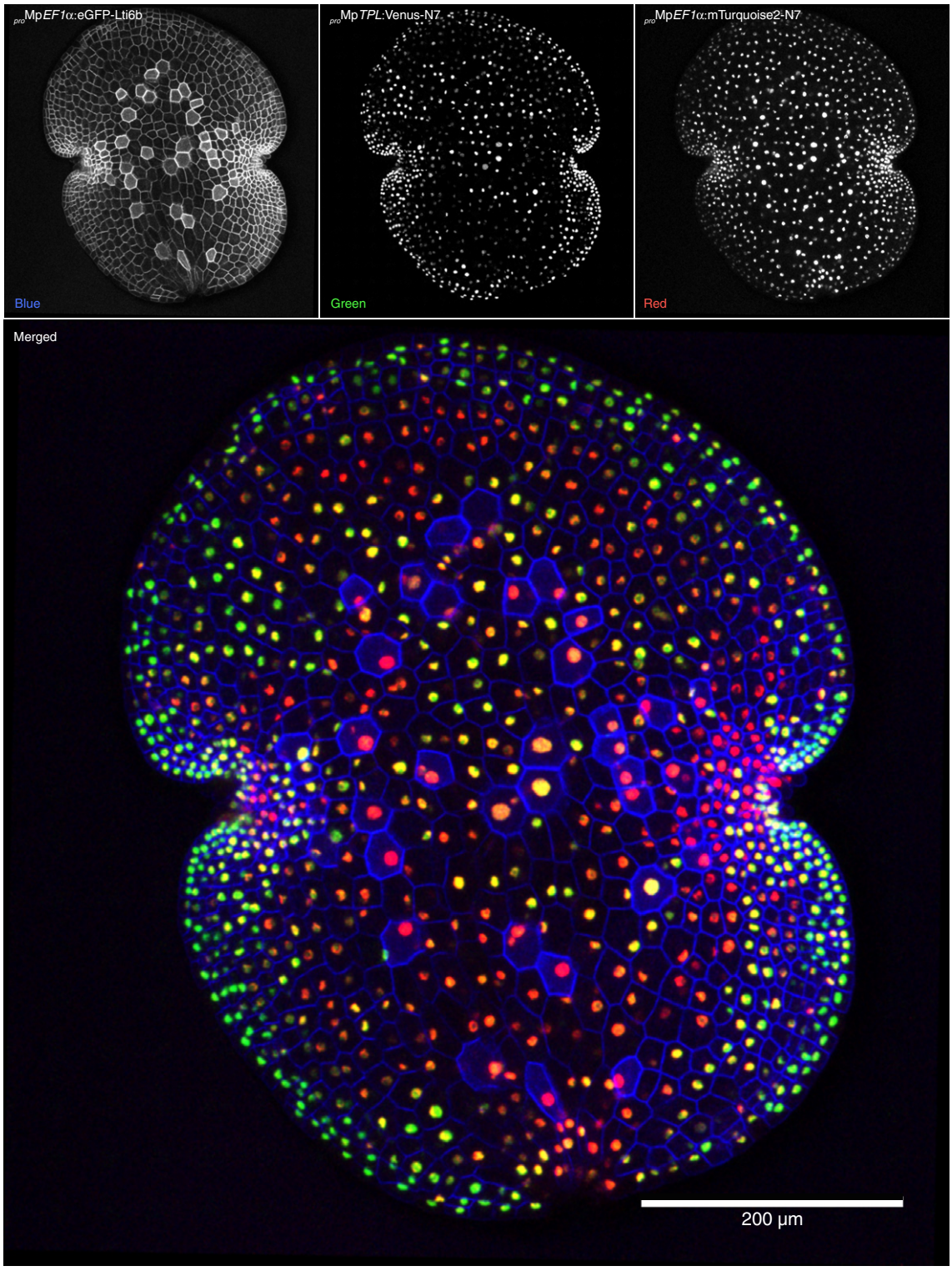

Fig. 5 *In planta* activity of a Loop assembly construct. *Marchantia gemmae* transformed with an L2 construct was imaged with a Leica SP8 laser scanning confocal microscope to assess the expression of fluorescent markers. mTurquoise2-N7, Venus-N7 and eGFP-Lti6b were excited with appropriate wavelengths and fluorescence was captured in their respective emission windows in sequential scanning mode. Images shown are Z-stack maximum intensity projections.

between digital design and experimentation. We have demonstrated the usefulness of LoopDesigner by implementing a simple web tool via which users can design assembly strategy and run virtual reactions before stepping into the laboratory. The LoopDesigner framework allows the definition of Loop assembly schemas of arbitrary complexity, with any number of levels and plasmids per level, as well as with any possible restriction enzymes and overhangs. In this sense, LoopDesigner generalizes the

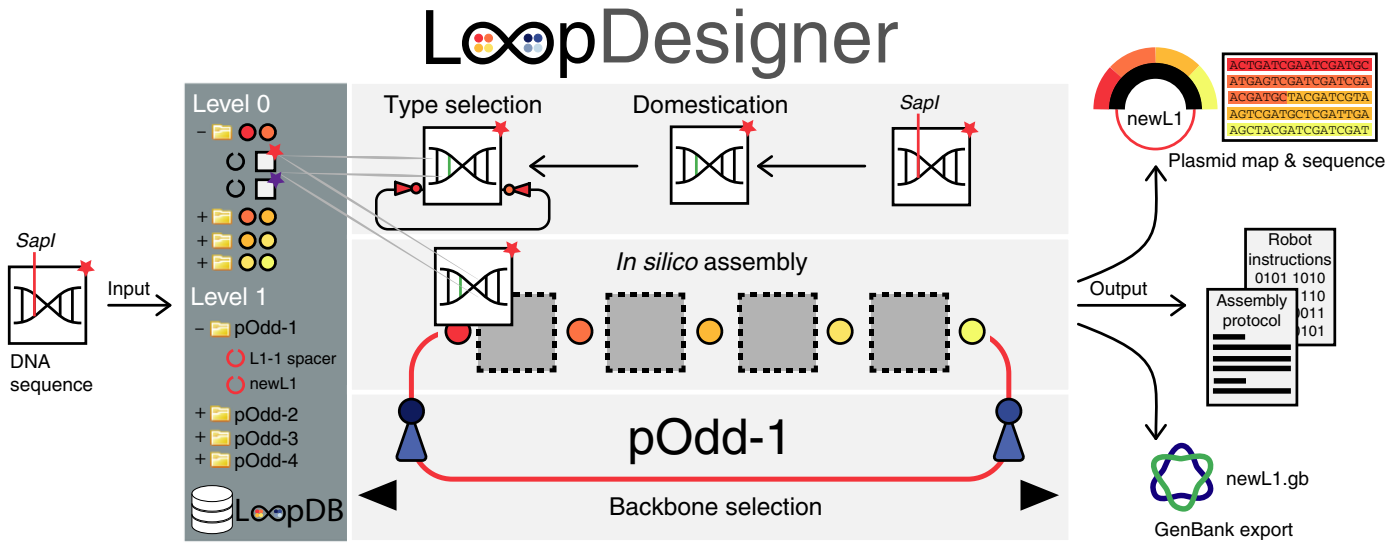

Fig. 6 Design automation. A DNA sequence is submitted to LoopDesigner, which screens for BsaI and SapI sites and domesticates them to silent mutations where possible. A *part* type is specified for the assembly schema to save the *part* to the database library. To perform an *in silico* assembly, a receiver plasmid is selected which displays the compatible *parts* that can be placed in the current position of the assembly schema. As *parts* are included, the next compatible *parts* are displayed. When the assembly schema finds that all the *parts* required to complete the assembly are selected, the assembly simulation is performed. Then, LoopDesigner outputs the resulting plasmid map with its concurrent highlighted sequence and a protocol for Loop Type IIS reaction setup or export of GenBank sequence. Instructions to robots can be outputted if an API is provided with the required information (plasmid positions, ID mappings, robot functions) to produce the concurrent instruction file using *Python* scripting. The assembled *part* is then saved into the *part* library database for further assembly.

concept of the assembly, so that the assembly schema presented in this article becomes a single instance of many possible implementations of the Loop assembly, allowing for the exploration of novel ways of assembling DNA parts through Type IIS strategies.

DNA construction has been traditionally coupled with the concurrent use of plasmids in model organisms. Loop assembly provides additional throughput and versatility for working with general-purpose backbones, to which users can add specific new functions, for example, parts for transfection. Vectors could be decoupled from specific uses by modularizing replication origins and selection markers as basic DNA parts and introducing host-specific elements during the assembly process. This would provide higher flexibility during design, and allow the switching of selection markers when super-transformation is required, for instance. Such approaches would make the DNA fabrication process host-agnostic, promoting the development of universal DNA assembly systems using standards such as the common syntax, which would provide unprecedented exchange of DNA components within the biological sciences.

Until recently, the majority of materials for research were exchanged under a Uniform Biological Material Transfer Agreement (UB-MTA). This is a bilateral legal agreement which, in its standard form, does not allow redistribution, exchange or use with those outside of educational and research organizations. At the same time, in scientific publishing and in software, there is a trend towards openness to facilitate collaboration and translation of basic research. An excellent example of how the open source philosophy has powered and enabled innovation is exemplified by community-based coding projects, such as those hosted by *Github* (https://github.com). *Git* was originally developed for the purpose of distributed software development, and nowadays most collaborative projects, both in the public and private sector, use *Git* as an underlying framework. It is unlikely that we will see similar success in DNA engineering and synthetic biology unless new forms of unrestricted DNA sharing and assembly are established under more open frameworks, such as the OpenMTA. We support the adoption of an open-source inspired L0 elemental part exchange by providing Loop assembly for the higher level construction of these L0 components under an OpenMTA framework. Work to establish the OpenMTA will ensure access to the Loop assembly system for work in both the public and the private sector.

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Author contributions

BP, FF and JH conceived the study. BP and FF developed the Loop assembly logic. BP designed and implemented the Loop assembly schema, built the Loop assembly library, optimized the reaction conditions, validated the recursive assembly and performed the characterization of Loop assembly efficiency. BP and MD built the Loop assembly constructs. TM, SA and FF designed the UNS for plants. SA performed preliminary experiments to validate the UNS-mediated assembly. AC assembled Loop constructs through UNS overlap assembly and collected expression data on *Arabidopsis thaliana* protoplasts. MD and BP designed LoopDesigner, and MD developed PartsDB and LoopDesigner. AW performed HT sequencing on Loop assembly constructs. NJP provided advice with regard to Type IIS restriction endonucleases. BP performed the *Marchantia* work and collected the microscopy data. FF and JH supervised the project. FF, RAG and JH secured funding for the development of the project. BP, MD, RAG, NJP, FF and JH wrote the manuscript. All authors revised the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 pOdd-spacer and pEven-spacer usage.

Fig. S2 Standardized unique nucleotide sequence (UNS) PCR.

Fig. S3 L3 assembly verification.

Fig. S4 Transient expression of a multi-transcription unit (multi-TU) L2 construct in Arabidopsis mesophyll protoplasts.

Fig. S5 Plasmid maps for L2 constructs used for plant heterologous expression.

Methods S1 Loop assembly unique nucleotide sequence (UNS).

Notes S1 Synthetic promoter assembly.

Notes S2 Characterization of Loop assembly efficiency.

Table S1 DNA parts.

Table S2 Loop Type IIS assembly DNA preparation.

Table S3 Loop Type IIS assembly reaction preparation.

Table S4 Loop Type IIS assembly cycling conditions.

Table S5 Unique nucleotide sequence (UNS) primers.

Table S6 Loop vector unique nucleotide sequences (UNSs) for plants.

Table S7 High-throughput sequencing validation of Loop assemblies.

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