DNA Assembly Standards: Setting the Low-Level Programing Code for Plant Biotechnology

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Abstract

Synthetic Biology is defined as the application of engineering principles to biology with the aim of increasing the speed, ease and predictability with which desirable changes and novel traits can be conferred to living cells. The initial steps in this process aim to simplify the encoding of new instructions in DNA by establishing low-level programming languages for biology. Together with advances in the laboratory that allow multiple DNA molecules to be efficiently assembled together into a desired order in a single step, this approach has simplified the design and assembly of multigene constructs and has even facilitated the automated construction of synthetic chromosomes. These advances and technologies are now being applied to plants, for which there are a growing number of software and wetware tools for the design, construction and delivery of DNA molecules and the engineering of endogenous genes. Here we review the efforts of the past decade that have established synthetic biology workflows and tools for plants and discuss the constraints and bottlenecks of this emerging field.

Keywords

synthetic biology, DNA assembly, molecular cloning, laboratory automation, biotechnology, genome engineering, CRISPR

Introduction

Plant synthetic biology applies top-down engineering to re-program plant cells with new genetic instructions, aiming for novel and favorable outputs. The use of plant species as so-called 'chassis' for engineering, allows synthetic biologists to take strategic advantage of the 450 million years of evolutionary forces that resulted in autotrophic, sun-fueled organisms with a surplus of reducing capacity that can be converted to food, feed, medicines, textiles and a range of other high-value products [1]. Proficient engineering of plant chassis is a captivating and challenging endeavor that may, arguably, become indispensable for the preservation of human life on earth, yet we have barely initiated the first, necessary steps.

Synthetic biology often uses computer engineering as an analogy that, although imperfect, serves to situate current efforts. In computer sciences, the lowest level in the programming code hierarchy is “machine code”, which can be equated to the universal ATGC genetic code, common to all known living cells (Fig. 1 A). While computer engineers can write programs in machine code, the process is tiresome and error-prone requiring a high level of expertise. For this reason computer programs in modern contexts are typically written using higher order languages. Similarly, programing genetic instructions at the nucleotide level would be a low-throughput and extremely time-consuming task and therefore higher abstraction levels become necessary.

The level above machine code includes the so-called low-level languages, which provide very little abstraction, maintaining a close relationship with the details of the machine. Low-level computing languages, also known as assembly languages, are each specific to a particular computer architecture. As in the computing analogy, low-level programs in synthetic biology are those using syntax elements that closely resemble the code that is physically (in this case, chemically), executed by the hardware (the cell). Thus, synthetic biologists define small functional DNA fragments such as promoters, ribosome binding sites (RBSs), coding sequences (CDSs), terminators, etc., as the basic elements from which transcriptional units, multigene devices and complex synthetic circuits can be assembled (Fig 1B, C). Low level Synbio codes also include the instructions and rules for the physical assembly of those basic DNA parts, which are equivalent to the enzymatic reactions executed in the cell, such as DNA ligation, digestion or recombination. Furthermore, as happens with computer assembly languages, DNA assembly codes need certain adaptations to suit a particular chassis architecture, whether prokaryotic or eukaryotic cells, plants cells, etc.

Further up the hierarchy, high-level languages such as C++, Python and Java are further abstracted, even employing natural language elements to ease their use. Synthetic biology aims to create similar abstraction levels for programming cells, where low-level programing codes are minimally abstracted from the base DNA code run by cells while further abstractions will expedite the programming of cellular functions (illustrated in Fig 1D). It is important to note that the integration of new code into an exceedingly complex environment evolved to perform specific functions that exert extreme interference on the new code are constraints that do not exist in bottom-up, human-made computers and hence the analogy becomes imperfect. Further, the establishment of a biological programming language requires a concerted effort to define syntax, vocabulary and assembly standards. Nevertheless, and despite the limited number of avenues currently available for delivering new information to plant cells, the rudiments of low-level codes for plants have recently been established, and as a consequence, our ability to program increasingly sophisticated novel genetic functions into plant cells has rapidly expanded. We review here the challenges of establishing low-level assembly codes for Plant Synthetic Biology, from early hardwired binary plasmids to the recent multipartite standards and common syntax.

Life before Standardization

Up until the beginning of the 21st century, most labs employed plasmids, often binary plasmids equipped with the necessary features for Agrobacterium-mediated transformation, for the delivery of transgenes to plant genomes. These contained polylinkers (multiple cloning sites) comprising recognition sites for various Type II restriction endonucleases (RE). These polylinkers were typically flanked by hardwired regulatory sequences, most often the 35S promoter from the Cauliflower Mosaic Virus and a terminator from the Agrobacterium *Nos* or *Ocs* gene, to direct constitutive expression of the coding sequence (CDS) cloned by the user into the polylinker. Non-constitutive transgene expression could be achieved by laborious stepwise cloning of different elements. These rudimentary tools limited most experiments to single-transgene constructs and multigene engineering was typically achieved by sexual crossing, sequential re-transformation, or co-transformation using two Agrobacterium strains with different T-DNAs [2,3]. Co-transformation was also particularly successful in biolistic transformation, where simultaneous delivery of DNA fragments resulted in the integration of several transgenes in a single event [4] . Several schemes for multigene engineering were

proposed using combinations of zinc fingers and homing endonucleases, *in planta* recombination, and the popular Gateway® cloning technology based on Lambda (λ) recombination between site-specific attachment (att) sites [5]. Gateway® technology introduced unprecedented efficiency and flexibility and, although they initially existed as hardwired single-purpose tools, the ability to convert new plasmids into destination plasmids, each designed for a specific “programing” goal such as overexpression, gene silencing or tagged expression was instrumental in their success [5–7]. Further progress in combinatorial flexibility was introduced with Multisite Gateway®, which incorporated an extended collection of att sites for combinatorial arrangements of up to four DNA fragments, opening the way for the definition of a rudimentary assembly syntax of building blocks such as promoters, CDSs and terminators [8]. This led to the creation of dedicated collections of Gateway® genetic elements for e.g. fruit biotechnology [9]. However, Gateway® had significant drawbacks: the limit of four elements for simultaneous assembly, a thirteen base pair so-called 'scar' between assembled sequences, and the relatively high-price of the proprietary reagents. While iterative assembly, alternating between 'BP' and 'LR' clonase reactions (named for the specific att sites to be recombined), allowed for more complex constructs [10], the proliferation of scars was undesirable and limited precise genetic designs. Subsequent technologies therefore focused on minimizing the presence of undesirable bases between assembled fragments and increasing the number of fragments that could be assembled in parallel.

Applying the principles of engineering to molecular cloning

Together with abstraction, the engineering principles of standardization and modularity have been applied to DNA fragments with the aims of overcoming limitations of previously cloning technologies and making engineering biology more predictable. The application of these principles have come to define the field of synthetic biology [11]. One of the first steps was the adoption of the design-build-test-learn (DBTL) cycle that typifies industrial processes. New genetic combinations are designed from standard parts and constructed into longer molecules using defined assembly standards for testing in the ‘chassis’. This workflow can be progressed iteratively, employing data from each test to inform improved designs. This improves both the performance of the device and, when coupled with systematic and standardized storage of data from the performance of standard parts, facilitates the design and build of further novel devices.

From its foundation, synthetic biology included the concept of standard DNA parts as building blocks with basic biological function [12]. The standardization of these basic building blocks

enabled the modularization of assembly, simplified laboratory protocols decreasing the effort required for the assembly of multigene sequences, and enabled parts to be easily exchanged and reused [13]. Assembly standards have reduced the requirement for expertise in multiple molecular techniques, as well as for large suites of reagents. The first DNA assembly method to utilize such parts was the BioBrick standard [14]. This proposed a sequential cloning scheme for the pairwise assembly of standard parts (BioBricks) to create multigene assemblies making use of four Type II REs: *Eco*RI, *Pst*I, *Spe*I and *Xba*I. Digestion of the destination vector and the donor insert with the appropriate enzymes generates complementary sticky ends that, when ligated, recreate the original sites flanking the new component and creating a six nucleotides scar at the junction. BioBricks are most widely used by microbiologists and by participants in the annual international synthetic biology competition iGEM (International Genetically Engineered Machine). This competition started in 2004 as an in-house competition at MIT with five teams and has continued to grow with more than 300 teams from over 40 countries participating in 2017. However, the sequential, pairwise nature of construction, while robust and facile for undergraduates, is time-consuming and the use of Type II REs results in scars, making it unsuitable for purposes such as protein engineering. Although Biobrick-compatible binary vector plasmids were constructed [15] the standard was never widely adopted by the plant community and the Biobrick parts repository (http://parts.igem.org/Main_Page) contains few parts for plants.

Scaling up build: The rise and rise of parallel assembly methods

While in previous decades, the assembly of large and complex molecules was complex, low-throughput work for a skilled molecular biologist, new approaches have made such tasks routine and accessible. Inspired by Synthetic Biology principles, a number of methods have been developed for the simultaneous assembly of multiple DNA fragments in a desired order in a single reaction. Such techniques have become known as ‘parallel DNA assembly methods’ and are particularly attractive for projects with large numbers of complex constructs and when serial or combinatorial approaches are required.

In the last decade, many methods for combining multiple inserts into a single plasmid have been developed and the repertoire of tools and techniques continue to grow. In particular, non-proprietary methods that either create and assemble linear, double-stranded, overlapping fragments of DNA [16–19]**,** or that use a particular type of restriction endonuclease (Type IIS) to assemble multiple fragments have become widespread.

Double stranded, overlapping linear fragments are typically produced by PCR amplification using long oligonucleotides in which a region of sequence from the fragment that will be adjacent in the final assembly is included in a 5' tail (Fig. 2 Ai). The most used method is known as isothermal or Gibson Assembly (after Dan Gibson, who developed the technique). In this reaction, the 5' ends are digested using T5 exonuclease to create 3' overhangs allowing the single-stranded homologous ends to anneal [16]. The overhangs are filled in using a high-fidelity polymerase and the remaining single-stranded nicks are mended using *Taq* DNA ligase, such that a closed, circular molecule is recovered for bacterial transformation (Fig. 2 Aii). The advantage of this method is that the three necessary enzymes can be combined in a cocktail with all DNA fragments allowing a one-pot reaction, which takes place at a constant temperature. As a drawback, separate PCR amplifications with new primers to introduce the required homology, must be performed for each fragment in each new assembly. Besides that, there are few restrictions on the sequences of DNA fragments or the accepting vector and the final assembly is free from any extraneous sequences, known as ‘assembly scars’, that would be left by traditional restriction-ligation cloning. However, there are design requirements for the overlap sequences, which must not have any significant secondary structure. Additionally, sequences that contain repeats and short fragments that the exonuclease may reduce to a single strand, can drastically reduce the efficiency [20].

In contrast, methods that utilize Type IIS restriction enzymes (also referred to as Golden Gate) can be used to assemble multiple fragments, even if identical, simultaneously [21]. However, prior to the assembly reaction, all DNA parts, including the accepting plasmid, must be domesticated. In DNA assembly jargon, domestication is the process of providing DNA parts with flanking recognition sites for selected Type IIS enzymes (typically BsaI), and removing internal instances of the recognition sites for the same enzymes (Fig. 2 Bi). While this is somewhat laborious, once a fragment or accepting plasmid is prepared, it can be reused in new assemblies without further modification providing that the overhangs produced upon digestion are compatible. Standardization of DNA parts with equivalent function (e.g. promoters, CDSs etc.) for Type IIS assembly has led to the production of interoperable suites of DNA parts (so-called phytobricks, see below) that can be exchanged and re-used in new assembles [22–24]. Such toolkits are discussed in further detail below. Like Gibson assembly, Type IIS mediated assemblies can be performed in a one-pot reaction containing all fragments to be assembled, the enzyme and T4 DNA ligase (Fig. 2 Bii).

Parallel assembly methods, combined with bacterial artificial chromosomes and yeast replicating plasmids, have enabled the construction of very large pieces of synthetic DNA. In 2008, researchers at the J Craig Venter Institute reported the synthesis, assembly and cloning of a 582,970 base pair *Mycoplasma genitalium* genome from synthetic overlapping oligonucleotides [25]. The oligonucleotides were iteratively assembled, first into 101 5-7 kb fragments, which were subsequently assembled into 24 kb, 72 kb, 144 kb ("1/4 genome") and finally a full genome. In 2010, they also reported the assembly of the 16.3 kb mouse mitochondrial genome from 600 overlapping 60 bp oligonucleotides [26]. In 2014, the first eukaryotic nuclear chromosome (the 316,617 base pair chromosome III of *S. cerevisiae*) was assembled by homologous recombination in yeast from ~2 to 4 kb so-called minichunks, themselves assembled from 60- to 79-mer oligonucleotides [27]. This was the first stage in the Sc2.0 project, of which the ultimate aim is a synthetic genome [28]. At the time of writing, two efforts to assembly synthetic plant chloroplast genomes are known to be underway (N. Stewart and J. Ajioka, personal communications), and a project to 'write' human chromosomes is being planned [29]. The main bottlenecks for the assembly of large, even genome-scale, synthetic DNA molecules, therefore, are no longer technical feasibility, but cost and our ability to (re-) design chromosomes that function *in vivo*.

Standard parts and toolkits for plants

When inserting multiple genes into a plant genome, it is usually desirable to deliver them together on a single DNA fragment to enable integration at a single genetic locus, thus preventing segregation of the transgenes in subsequent generations. As described above, Type IIS-mediated DNA assembly facilitates the design of multigene constructs and allow the generation of standard and, therefore, interoperable parts. With the aim of facilitating exchange and re-use of DNA parts, several toolkits were created to enable the rapid assembly of such parts into binary plasmid backbones for delivery to plants. The availability of such toolkits comprising not only standard parts and vectors, but also information about part performance and reliable assembly, was key to their uptake, particularly by collaborating laboratories. To enable broader exchange across the plant community, in 2015 an agreement for a so-called common assembly syntax for plants was made the phytobrick standard), allowing compatible parts to be assembled using a number of Type IIS-mediated plasmid toolkits [24,30]. These include the Modular Cloning (MoClo) [31,32] and GoldenBraid (GB) toolkits [33–35]. While MoClo allows the parallel assembly of transcription units into multigene constructs, speeding up the cloning process, GB uses a pairwise method, instead favoring the reuse of intermediate

assemblies resulting in smaller, simplified set of backbones and tools. Further, the use of GB is supported by a web-based platform that includes software tools to facilitate part design, assembly and the storage of experimental data [35]. Facilitated by plasmid repositories such as Addgene (https://www.addgene.org), a large number of basic parts, compatible with both systems, have been made available including regulatory sequences, reporters, antigenic and sub-cellular localization tags and selectable marker genes [32,34].

The modular nature of these toolkits has facilitated the adoption of genome engineering technologies in plants through the addition of 'expansion sets' with parts for programmable nucleases and associated sequences [36,37]. These include tools derived from Transcription Activator-Like Effector Nucleases (TALENs), of which the DNA recognition domain can be recoded to recognize almost any sequence, as well as those from bacterial CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) systems that require a Cas (CRISPR-associated) nuclease to be expressed in combination with a guide RNA that recognizes the genomic target. Being facile to reprogram, CRISPR-derived tools have become particularly widely used. However, the construction of both TALENs and RNA-guided Cas constructs require the assembly of multiple parts. To construct a new TALEN, the specific 33-35 amino acids repeats within the DNA-binding module must be assembled, while in CRISPR-derived system, one or more guide RNAs must be co-expressed with the Cas protein. The repeated elements present in these assemblies are problematic for overlap-dependent assembly methods and, therefore, Type IIS methods have been frequently employed. The MoClo TALEN toolkit was one of the first kits dedicated to genome engineering in plants and offers a collection of DNA parts for constructing TALEN transcriptional units in a single step [38,39]. Similarly, the earliest reports of Cas-mediated genome engineering in plants employed the MoClo toolkit [40]. Subsequently, the use of the MoClo [41,42], GB [43] toolkits, as well as novel Type IIS-mediated systems [37,44–46], for DNA assembly has enabled genome engineering in number of different plant species.

Automating the build process

The primary costs of assembling DNA, especially as we move towards an era of synthetic genomics, are those associated with the purchase of synthetic DNA molecules, and those associated with constructing and sequence-verifying the assemblies. The costs of the former

are expected to fall as the industry continues to innovate and market competitiveness increases. It is also likely that the current limitations on molecule length will be reduced as providers integrate assembly into their portfolio of services. In the meantime, the cost of assembling DNA fragments can be reduced by increasing throughput and reducing input by miniaturizing reaction volumes. Both of these can be achieved using laboratory automation. Indeed, several institutions across the USA, Europe and Asia have now established facilities, known as DNA Foundries, dedicated to automating the design, construction and characterization of fabricated DNA. In particular, the use of acoustic dispensing technologies to transfer nanoliter-scale liquid droplets with high precision and accuracy has enabled DNA assembly reactions to be set-up at the nanoliter scale, cutting reagent cost by up to 100-fold [47]. In DNA Foundries, automated assembly has been coupled with robotic platforms for colony picking and purification of plasmid DNA, reducing the effort of the entire cloning workflow. In addition, the cost of assembly verification and rigorous quality control has been implemented by replacing Sanger sequencing with automated, highly multiplexed next-generation sequencing protocols [48,49]. While the majority of automation in synthetic biology has taken place on non-specialized liquid handling platforms, there has also been a sustained effort to enable workflows on programmable microfluidic platforms that offer a lower entry price point for automation [50]. In yeast and bacteria, microfluidic ‘lab-on-a-chip’ have been developed for an end-to-end workflow from transformation to automated gene expression induction, phenotype screening and measurement of metabolites [51]. The costs of miniaturized experimentation may be reduced even further by 3D-printed and open-source microfluidics on which DNA assembly reactions have already been successfully automated [52,53]. While there have been several reports of capturing of individual plant protoplasts in microfluidic chambers or spherical hydrogel beads [54,55], automated, high-throughput platforms for the assembly, delivery and characterization of synthetic DNA molecules in plant cells remains to be fully implemented.

Registries and repositories

Synthetic biology now aims to further develop part registries (data) and repositories (DNA). Registries manage the functional documentation of parts, from basic sequence data to functional descriptions, ideally provided by the registry's user-community. The Registry of Standard Biological Parts, founded at MIT in 2003, pioneered this venture introducing the Biobricks assembly standard and serving as a basis for the iGEM competition. More recently, plant-dedicated registries have developed by individual labs and consortia e.g. (https://gbcloning.upv.es). While these are better adapted for plant biotechnology, because they

tend to be bound by a single assembly standard, they miss the advantages of integrating the knowledge gathered by the breadth of the plant community. Ideally, to accelerate the engineering cycle, registries should incorporate parts compatible with multiple different assembly standards. In addition, concerted efforts will be required to develop also common rules for part documentation. This will enable developers to design higher-order programing codes that compile physical assembly rules and standard descriptions of registry parts, incorporating biological function as the next abstraction level.

Looking to the future - methods to accelerate the DBTL cycle

The norms for physical storage and exchange of biological materials are also changing in response to the establishment of repositories, the decline in cost of DNA synthesis and the administrative demands of material transfers. Currently, the most popular DNA repository is Addgene, a non-profit initiative that is fostering and facilitating the exchange of plasmids [56]. Repositories face the additional challenge of dealing with the property (including intellectual) rights of donors as well as limiting liability from the use of the materials by recipients. The most-used agreement for the exchange of biological materials, the Uniform Biological Material Transfer Agreement, prevents either redistribution of materials or the distribution for non-research purposes. A new agreement, the OpenMTA, aims to simplify and reduce the administrative burden of exchanging materials by supporting both redistribution, and the transfer of material between researchers at all types of institutions, while preserving attribution and the limitation of liability (Kahl et al., in review; https://biobricks.org/openmta/). Looking to the future, it could be argued that, given the continuous decrease in DNA synthesis prices, investment in physical repositories is questionable. However, the extremely low costs per base-pair of nanoscale automated assembly of existing DNA parts are unlikely to be breached for some years and therefore ready access to public libraries of standardized, characterized DNA remains desirable, particularly for those conducting research in low-resource regions for whom *de novo* synthesis remains out of reach.

The challenge of standard part documentation.

The success and growth of engineering in the 19th and 20th centuries can, at least partly, be attributed to the principles of modularization, abstraction and standardization for enabling incremental innovations. The properties of component parts were described and these operative and structural features were documented in detail and, critically, this documentation was made widely available in standard formats. As discussed above, to emulate the successes observed

in mechanical and electronic engineering, it is desirable to accelerate and scale the DBTL cycle by applying automation, and this is facilitated by the adoption of standards, both for the DNA molecules themselves also of the information held about these molecules. In the last few decades, we have seen an evolution in the standards for the storage of DNA information. The Genbank flat file, developed by the U.S. National Centre for Biotechnology Information (NCBI) and widely used by public sequence databases, is probably the most recognized file-type for genetic sequence information. However, although able to store metadata such as annotations, comments and references, this format was developed prior to the rise of synthetic biology and does not fulfil the requirements for storing data associated with assembly design and standards or characterization. To address this and to establish computational standards in synthetic biology, the Synthetic Biology Open Language (SBOL) was launched [57]. SBOL was created using a new terminology adapted to the needs of synthetic biology, providing file structures that incorporate fields for the inclusion of experimental results. A later iteration, SBOL v2.0 [58], incorporates both structural and functional design features and integrates systems biology modelling standards such as the System Biology Markup Language (SBML), linking computational models to wet-lab experimentation. SBOL is further defined by a set of symbols, SBOLv, to standardize the visual representation of standard DNA parts [59]. SBOL and SBOLv symbols for basic genetic parts have been integrated into several tools and standards for synthetic biology, including the GoldenBraid 3.0 standard for plants (Fig. 3 A) [35].

As the focus moves towards parts documentation, the consensus on how to integrate biological data for the functional characterization of parts and devices becomes more important. SBOL does not currently represent all types of biological design data, since many of these data types lack a clear consensus on their proper representation. It allows however to embed custom data within SBOL objects and documents, enabling new types of data to be easily incorporated into the SBOL standard once there is community consensus on their representation (Fig. 3 B) [60]. To establish consensus on how to represent biological data even for the most basic phytobricks, such as e.g. the transcriptional activity of a plant promoter, is a challenge for experimentalists. Although standard measurements have been proposed in bacteria as for the transcriptional activities conferred by promoters and/or terminators [61–63], unfortunately these are difficult to implement in multi-celled organisms. Traditionally, functional information on regulatory elements in plants was obtained (and documented) by extrapolating the expression data of the regulated gene. In this sense, gene expression atlas developed in a few model species provide a fair indication of the average transcriptional activities provided by the regulatory regions flanking a

coding region [64,65]. However, this approach in imprecise as it often misses long distance cis-acting regulatory regions as well as epigenetic and positional effects, not to speak about the chassis-specificity of part and devices. To avoid this, it is desirable to characterize synthetic elements in contexts different from their native environment. In plants, a suitable option is to define semi- controlled environments and standard measurements for parts characterization, such as transient assays (protoplast transfection or leaf agroinfiltration) and reporter activities e.g. luciferase [35,66]. Transient assays enable quick, combinatorial and multiplexed part characterization, and facilitate rapid building of parts collections. For instance, normalized reporter activities have been employed to classify synthetic promoters on the basis of their relative transcriptional rates [34,67]. Unfortunately, quantitative characterizations with transient methods are not precise enough to sustain fine tune design in stable plants, however they are useful as guides for designers, which can make “educated” decisions based on standardized transient data.

Standardization and characterization in a genomic context

In most cases, the ultimate goal of a synthetic construct is its integration in the plant genome. Unfortunately, part characterization with transient assays do not integrate the genomic context. It is well known that the site of integration of a construct in the genome greatly influences transgene behavior [68], and this limiting the use of transient assays for fine tune characterization of synthetic devices. A solution long used in microbial biotechnology [69] is the identification of neutral integration sites to be used as consensus sites or “landing pads” for part/device characterization and engineering. Until recently, the low efficiency of homologous recombination in plants did not allow the designation of native landing pads at neutral sites in the genome. Instead, landing pads have been created in crop species for gene pyramiding approaches using site-specific recombination sites, playing a similar role as recombination target sites used in yeast genetic engineering. However, with increasingly efficient nuclease-based (e.g. CRISPR/Cas9) gene targeting strategies in place [70–72], the original landing pads concept can be re-oriented towards the definitions of hot spots for building synthetic genetic devices, from single transgenes to complex metabolic pathways, genetic circuits, etc. Theoretically, gene expression parameters can be characterized more precisely using these well-defined genomic context and with the assistance of easily-scorable reporters. Later, reporters can be substituted by genes of interest using precise gene targeting strategies. Although part characterization in specially-designated neutral sites is necessarily chassis-

specific and therefore more labor intensive than transient expression, this is probably the only valid option for implementation of genetic devices requiring fine-tuned control of transcriptional activities such as transcriptional gene circuits.

Concluding remarks

In just a few years modular and standardized DNA assembly methods have revolutionized genetic engineering in plants, enabling the construction of complex biosynthetic pathways [73–75], the design of challenging biofactories [76,77], or more recently, contributing to accelerate plant breeding with multiplexed genome editing [37]. All these examples of engineering projects would have been almost impossible to achieve without modern DNA assembly tools. By establishing consensus on how to use and exchange these tools, plant biotechnologists are founding the low-level programing codes in plant Synthetic Biology, which are contributing towards even more ambitious projects such as the creation of crop plants with improved tolerance to stress [78], C_4 rice with more efficient photosynthesis [79] or cereals with nitrogen-fixing ability [80]. The continuous decrease in the price of DNA synthesis and the advances in automation can only accelerate the advancement of our engineering capacities in the years to come. To take maximum profit of new technologies, new concerted efforts will be required to secure and reinforce the development of DNA assembly codes, safeguarding the confluence with standardization initiatives in other SynBio fields, but at the same time ensuring that the singularities of plant biotechnology are properly addressed.

Figure Legends

Figure 1. A plant genetic circuit described using four different levels of abstraction. The circuit measures the transient activation of a promoter (DFR) when connected to a Glucocorticoid sensor. It comprises a Dexamethasone (DX) sensor, a Myb-bHLH (Rosea1-Delila) responsive Luciferase (LUC) reporter, a Renilla reporter for signal normalization and the P19 silencing suppressor. (A) Machine code-like FASTA file; (B) Graphical description using SBOLv adapted to Level 0 (basic) phytobricks; (C) Similar representation using Level 1 gene modules (transcriptional units) enriched with functional interactions; (D) Hypothetical higher level representation. Level A resembles a computer machine code, levels B and C are equivalent to low-level assembly codes and D illustrates higher level abstraction codes.

(Figure 1.pptx)

Figure 2. (A) Overlap-dependent and (B) Type IIS-dependent methods for parallel assembly of multiple DNA fragments (Ai) PCR amplification using long oligonucleotides to introduce a region of homology with the adjacent fragment in a 5' tail results in a set of linear, overlapping double-stranded DNA fragments. (Aii) These are assembled in an isothermal reaction with a plasmid vector linearised with a restriction endonuclease or by inverse PCR. All components are combined with a three enzyme cocktail: T5 exonuclease, performs a 5'-3' resection of all linear DNA molecules allowing the complementary overhangs to self-anneal, a proofreading DNA polymerase fills in the gaps and *Taq* ligase repairs the nicks resulting in a circular molecule. (Bi) The recognition sequences of Type IIS restriction endonucleases e.g. *BsaI* are non-palindromic and therefore directional, they also cut outside of their recognition site.
Amplification (or synthesis) of DNA fragments introducing a pair of flanking convergent recognition sites for a Type IIS restriction endonuclease. These fragments can be cloned into an accepting plasmid for maintenance and reuse in multiple assemblies (Bii) Providing compatible overhangs are produced by digestion and the absence of internal instances of the recognition enzymes, DNA fragments flanked by a pair of convergent Type IIS restriction enzyme recognition sites can be assembled in a single digestion-ligation reaction into an acceptor plasmid with divergent Type IIS restriction enzyme recognition sites. This is possible because the resulting assembled molecule does not contain any of the target Type IIS recognition sequences and cannot be re-cleaved by the nuclease.

(Figure 2.pptx)

Figure 3. Standard DNA parts representation and documentation. (A) vSBOL-based symbols adapted to plant DNA parts. All subparts conforming a transcriptional unit have their associated symbol based on their function. Parts with combined functions as promoters followed by a 5’ untranslated region (UTR), entire coding sequences (CDS) or terminators preceded by a 3’ UTR also have their own symbols. (B) Example of a standard datasheet including general information of a DNA part (in blue), sequence and assembly data (in green) and experimental information derived from standard characterization (in orange).

(Figure 3.pptx)

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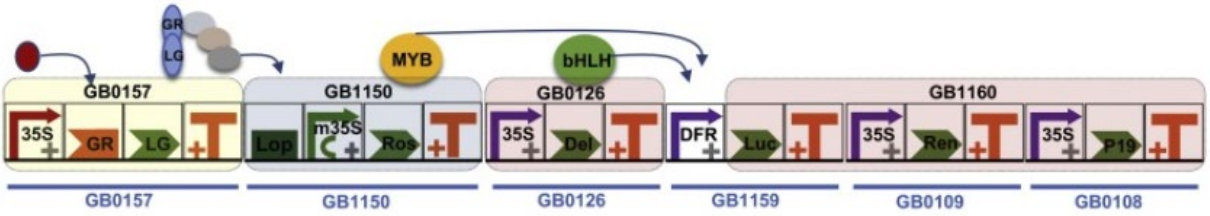
B

C



D

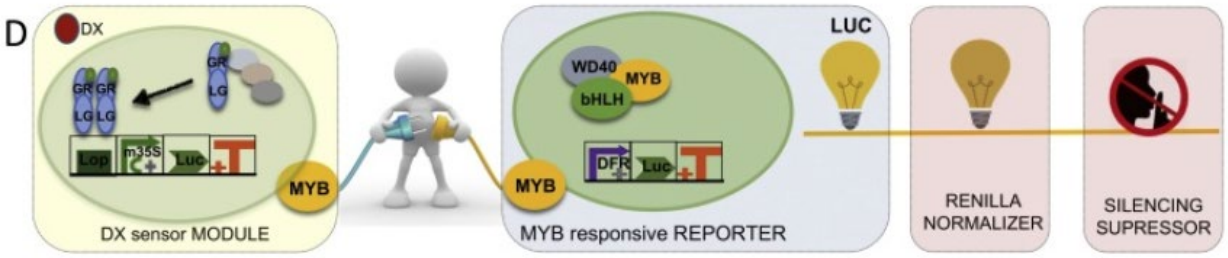


Fig 1

Fig 2

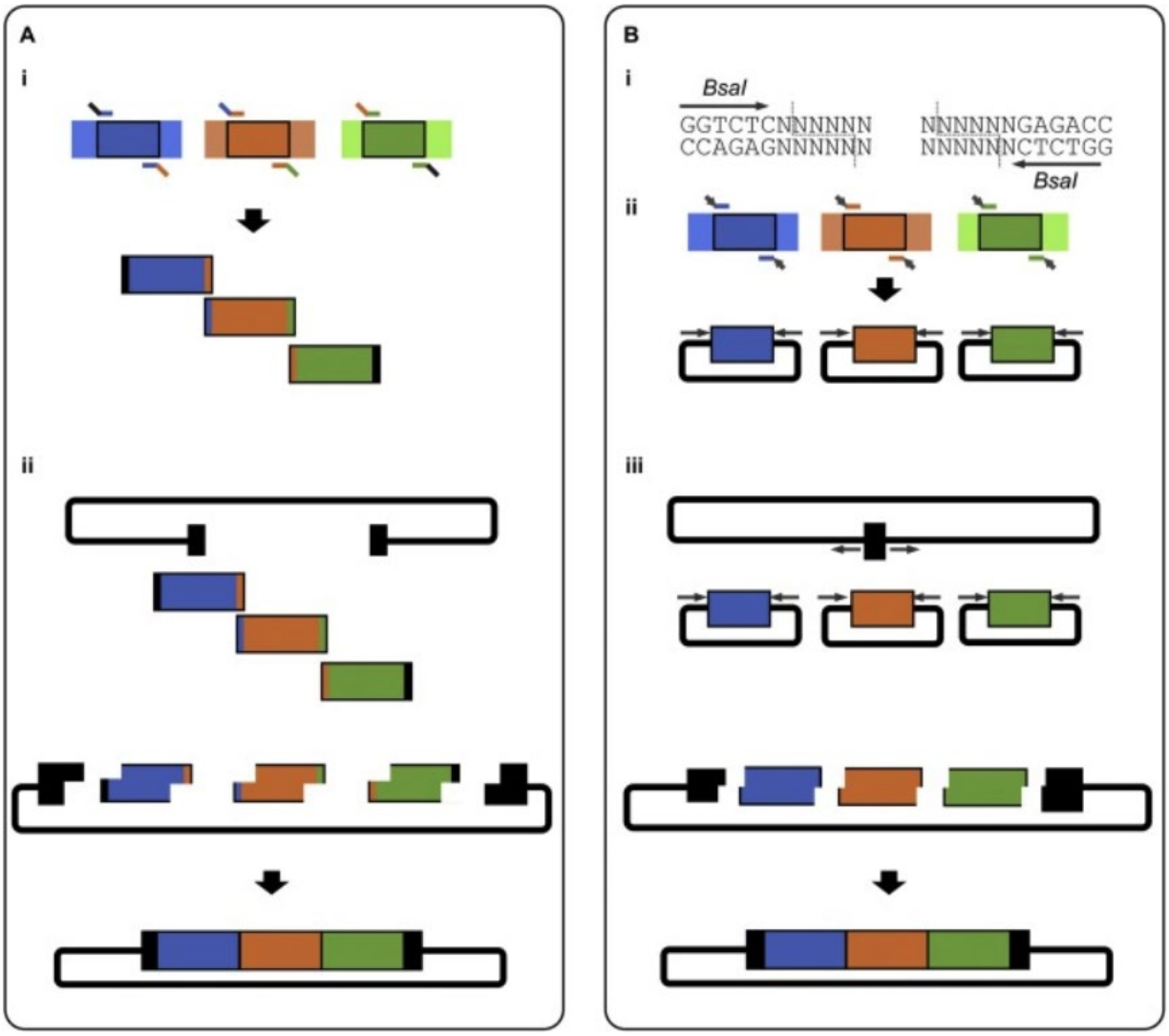
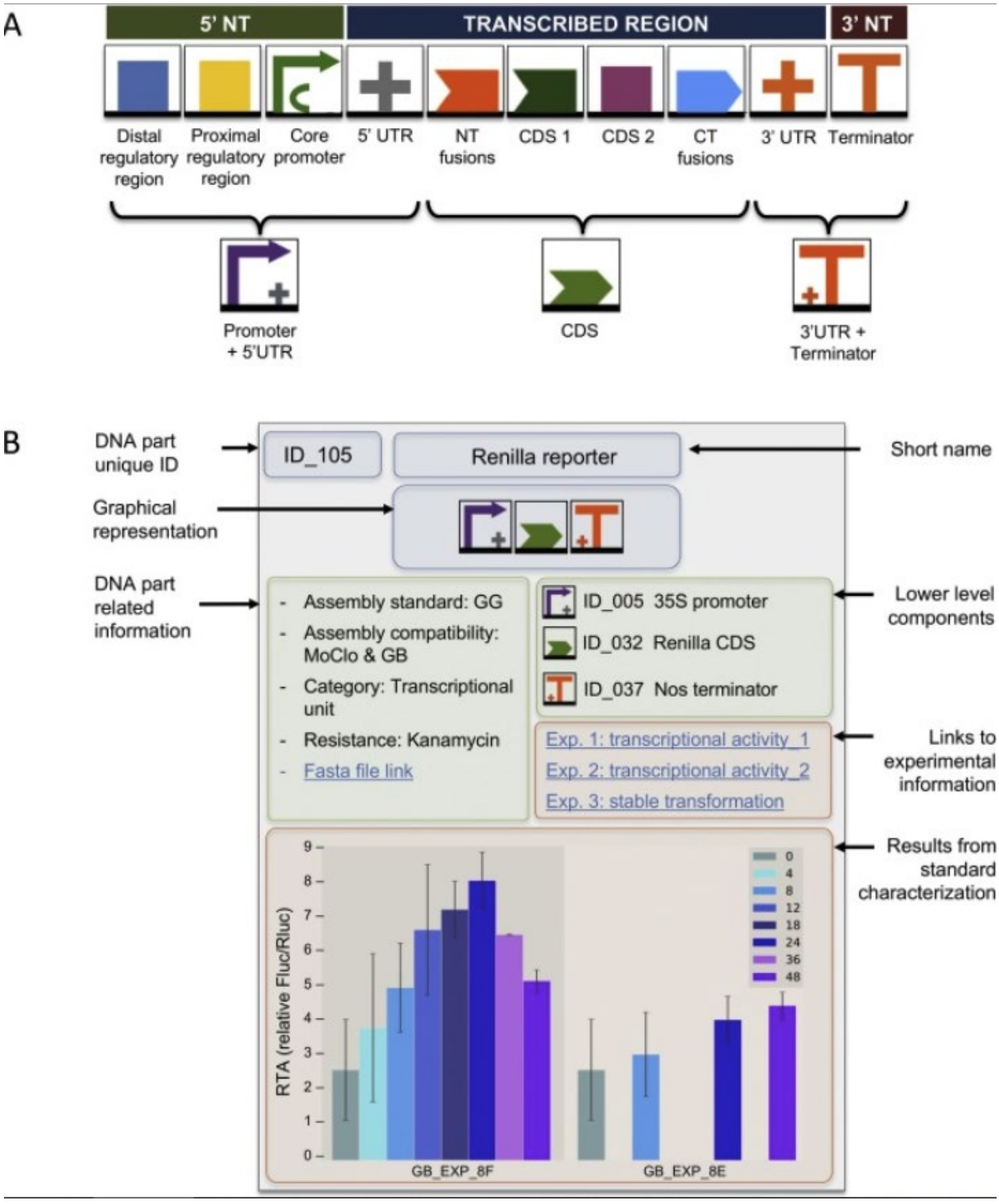

A
i
ii
B
i
BsaI
GGTCTCNNNNNN
CCAGAGNNNNNN
NNNNNNGAGACC
NNNNNNCTCTGG
BsaI
ii
iii

Fig 3


A
5' NT
TRANSCRIBED REGION
3' NT
Distal regulatory region
Proximal regulatory region
Core promoter
5' UTR
NT fusions
CDS 1
CDS 2
CT fusions
3' UTR
Terminator
Promoter + 5'UTR
CDS
3'UTR + Terminator
B
DNA part unique ID
ID_105
Renilla reporter
Short name
Graphical representation
DNA part related information
- Assembly standard: GG
- Assembly compatibility: MoClo & GB
- Category: Transcriptional unit
- Resistance: Kanamycin
- Fasta file link
ID_005 35S promoter
ID_032 Renilla CDS
ID_037 Nos terminator
Lower level components
Exp. 1: transcriptional activity_1
Exp. 2: transcriptional activity_2
Exp. 3: stable transformation
Links to experimental information
RTA (relative Fluc/Rluc)
0
4
8
12
18
24
36
48
GB_EXP_8F
GB_EXP_8E
Results from standard characterization