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The plant Golden Gate toolkit: compatibility, interoperability, and harmonized assembly in plant synthetic biology

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Title: The Plant Golden Gate Toolkit: Compatibility, Interoperability, and Harmonized Assembly in Plant Synthetic Biology.

Running head: A Guide to Golden Gate Plant Cloning Systems.

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Abstract

In plant synthetic biology, the emergence of Golden Gate (GG) cloning has led to the rapid development of numerous cloning kits that enable the standardized assembly of genetic modules and transcriptional units through a single, one-pot reaction. However, this rapid and somewhat

uncoordinated expansion has introduced challenges, particularly regarding compatibility between cloning kits, which can hinder adoption and routine use in new laboratories. To address these recurring issues, this review provides a comprehensive overview of more than 25 well-established plant-specific GG cloning kits, along with an in-depth examination of the compatibility of their available modules, with the goal of improving interoperability across systems. To support a more integrated and forward-looking development of future GG-based efforts, we present key approaches for increasing system harmonization, including several domestication strategies, methylase-assisted hierarchical DNA assembly, and the inclusion of multiple cloning sites. As a whole, this review aims to streamline cloning workflows and reduce technical barriers for new plant synthetic biologists in pursuit of increasing the throughput of their experiments.

Keywords

Plant synthetic biology, Golden Gate cloning, Modular cloning, Hierarchical cloning, Cloning system compatibility, Type IIS enzymes

Advances box

- New cloning frameworks (e.g., Modular cloning and Joint modular cloning) now support mixing linear additive and multiplicative workflows, allowing researchers to flexibly scale between simple transcriptional units and large multigene constructs in plant systems.
- Increasing availability of cross-compatible vector chassis (e.g., PhytoBrick syntax) makes it easier to transfer plant parts between different Golden Gate ecosystems.
- Refined strategies for protecting functional noncoding regions (e.g., methylase-based cloning) now allow the inclusion of plant regulatory elements (e.g., promoters,

terminators, and UTRs) that were previously avoided due to undomesticatable sites that require sequence integrity for specific experimental designs (e.g., functional validation).

- Improved domestication workflows now integrate low-cost DNA synthesis, high-fidelity PCR editing, site-directed mutagenesis, and phylogenetic search, to efficiently eliminate Type IIS sites in noncoding and coding sequences.

Box 1 – Terms and Definitions of Golden Gate Cloning

Golden Gate cloning

Cloning strategy that relies on Type IIS restriction enzymes such as BsaI and PaqCI, which cut outside their recognition sequence to generate user-defined overhangs for directional assembly. As defined in Patron et al. (2015), five general guidelines chaperone the use of these enzymes:

(i) Each genetic module is maintained in a plasmid vector flanked by a convergent pair of Type IIS restriction sites;

(ii) The destination (acceptor) plasmid contains a divergent pair of recognition sites for the same Type IIS enzyme, defining the locus at which one or more parts will be assembled;

(iii) Both the parts and all plasmid backbones are fully domesticated, meaning they do not contain any additional recognition sites for the Type IIS enzyme used in the assembly;

(iv) No part is carried on a plasmid backbone that shares the same antibiotic resistance marker as the destination plasmid into which the part(s) will be assembled;

(v) The overhangs generated upon digestion with the Type IIS enzyme are required to be unique and non-palindromic to ensure directional and specific assembly.

DNA modules (or ‘‘parts’’)

Discrete, standardized DNA elements (e.g., promoters, coding sequences, tags, or terminators) that are flanked by defined Type IIS restriction sites to enable their precise and modular assembly in Golden Gate cloning. Each module carries unique overhangs that specify its functional position in a construct, allowing interchangeable, mix-and-match assembly within a shared syntax. As described by Grützner and Marillonnet (2020), generating such standardized parts with polymerase chain reaction (PCR) typically involves a five-step workflow: (1) identifying the type of basic part to be cloned, (2) designing the corresponding primers, (3) amplifying the fragment, (4) assembling it using Golden Gate cloning, and (5) verifying the final construct by sequencing. While PCR is the most commonly employed method to generate DNA modules, alternative approaches such as DNA synthesis or oligo stitching can also be used.

Transcriptional units

Composite assemblies generated by joining multiple modules (e.g., promoters, coding sequences, and terminators) into a single expression cassette. In Golden Gate systems, transcriptional units are typically assembled using a different Type IIS restriction enzyme than the one used for Level 0 parts (e.g., BsaI for Level 0 parts, BpiI for Level 1 transcriptional units). These transcriptional units serve as intermediate building blocks that can be further combined via linear additive and multiplicative assembly strategies to generate higher-order, multigene constructs.

Dummy sequence

A neutral, non-coding DNA fragment used as a structural component in a cloning construct to aid assembly and preserve the intended design. A dummy sequence is characterized by biological neutrality (i.e., lacks the structural motifs required for transcriptional expression) and stability (i.e.,

avoid repetitive or unstable sequences comprising plasmid integrity) and aims to act as a placeholder or spacer to facilitate assembly and maintain the desired architecture. Dummy sequences can used in any type of cloning, but need to be carefully designed.

Box 2 – Definitions and Strategies for Golden Gate Domestication

General definition of Golden Gate domestication

Golden Gate domestication is the process of modifying a DNA sequence to remove internal recognition sites in sequences for Type IIS restriction enzymes, typically through silent or non-disruptive mutations. Sequence domestication is generally performed on coding sequences, where synonymous recoding (e.g., ACA can be modified to ACC as both encode a threonine amino acid) can eliminate unwanted sites while preserving the original biological function. In contrast, domestication cannot be applied to regulatory elements such as promoters and terminators when sequence integrity must be preserved (e.g., for functional validation), as even minor modifications can lead to unintended functional alterations. Although these elements can technically be domesticated, such alterations may affect expression levels, timing, or regulatory interactions, and therefore require careful validation to ensure functional integrity. While recent evidence suggests that synonymous recoding can still influence gene expression and, in some cases, protein structure [3], it remains one of the few practical strategies available for removing internal recognition sites while preserving coding sequences.

Partial Domestication

Partial domestication refers to the removal of *some*, but not all, internal recognition sites for a given Type IIS restriction enzyme within a DNA sequence. The remaining "illegal" sites may still restrict one-pot assembly. *For example, a coding sequence in which two of three internal BsaI sites are removed while one remains is considered partially domesticated for BsaI.*

Full Domestication

Full domestication is the complete removal of *all* internal recognition sites for a specific Type IIS restriction enzyme from a DNA sequence. This ensures that the part can be safely used in any Golden Gate reaction involving that enzyme, maintaining strict one-pot, one-tube assembly compatibility. *For instance, a coding sequence in which the only internal Esp3I site has been eliminated is fully domesticated for Esp3I and safe for any Esp3I-based Golden Gate reaction.*

Super-Domestication

Term initially coined to highlight the use of molecular tools (e.g., genetic engineering, marker-assisted selection, and genomic prediction) to conduct precise genetic modifications that enhance the crops' performance and yield beyond the limits of natural variation and standard crossbreeding patterns [4,5]. Super-domestication in the Golden Gate context describes the elimination of internal recognition sites for *all* major Type IIS enzymes used across Golden Gate–based cloning systems. This level of domestication maximizes module interoperability, allowing a DNA part to function seamlessly in multiple GG syntaxes and enabling broad compatibility across diverse synthetic biology workflows. *For example, a coding sequence engineered to remove sites for all major seven recognition sequences (Table 1) becomes universally compatible with most plant-focused Golden Gate toolkits. It is important to note that this set of recognition sites may expand over time as new enzymes are discovered.*

Box 3 – Standardized Parts for the MoClo System

MoClo Plant Parts I

The Plant Parts I kit provides a foundational collection of 96 standardized Level 0 modules designed for building multigene constructs for plant transformation [6]. The kit includes promoters (e.g., different versions of CaMV35S, AtNOS, and AtRbcs), UTRs (e.g., TMV, and PVX), tags (e.g., 3xFLAG), localization signals, reporters, selectable markers (e.g., BAR), terminators, and specialized modules (e.g., silencing suppressor), thereby allowing diverse expression designs validated in *Nicotiana benthamiana*.

MoClo Plant Parts II

Plant Parts II extends the MoClo ecosystem with infrastructure vectors that facilitate transfer of PhytoBrick modules into other systems such as Gateway, yeast two-hybrid, and bacterial type III secretion [7]. It adds 80 new Level 0 PhytoBrick modules, compatible with both MoClo and GoldenBraid. Key innovations include GG-compatible Gateway entry vectors (pJOG130/131), MoClo-adapted Y2H vectors, infection-based secretion plasmids (pCK013–016), and a VIGS vector (pTRV2-GG), all assembled using BsaI.

MoClo Plant Parts III

Plant Parts III provides 94 new Level 0 modules for plant transformation and genome engineering [8]. The regulatory elements include a diverse set of promoters, terminators, and insulators. The coding sequence collection provides a range of functional modules, including site-specific

recombinases, fluorescent and luminescent reporters, metabolic reporters, selectable markers, developmental regulators, and the Cas9 nuclease.

MoClo CRISPR/Cas Toolkit for Plants

This kit provides 95 plasmids for assembling large CRISPR/Cas constructs featuring diverse nucleases (SpCas9, SaCas9, Lb/FnCas12a), base editors, and the EvolvR mutagenesis system [9,10]. It allows up to 24–30 gRNAs by combining tRNA–sgRNA arrays or individual Pol III-driven units in Level 2 vectors.

zCas9i MoClo-Compatible Kit

The zCas9i toolkit provides an intron-optimized, intronized maize codon-optimized Cas9 gene designed to improve nuclease expression and editing efficiency in plants. It supports hierarchical assembly of up to 32 sgRNAs using "multi-multi'' vectors, enabling high-level multiplex genome editing within a MoClo-compatible framework [11,12].

MoBiFC Toolkit

The MoBiFC toolkit adapts MoClo for bimolecular fluorescence complementation by assembling fluorescent protein fragment–proteins of interest fusions that enable visualization of protein–protein interactions in chloroplasts. It includes 23 Level 0 modules and validated positive and negative controls, providing a standardized system for chloroplast-targeted interaction assays [13].

MoChlo Chloroplast Engineering Toolkit

MoChlo provides 128 chloroplast-specific modules, including numerous promoters, UTRs, and destination vectors tailored for operon-based metabolic engineering, using BsaI/BpiI for Level 0 and Level 1 assembly [14].

1.0 Introduction

The development of modular Golden Gate (GG) cloning systems has revolutionized the process of genetic engineering as scientists can now assemble complete transcriptional units (TU) from a common set of genetic components (e.g., promoters, untranslated regions/UTRs, coding sequences, and terminators) [15]. To do so, GG cloning relies on Type IIS restriction enzymes which have the unique feature of cleaving DNA targets that are located outside their recognition sites, thus creating a large variety of overhangs that can be synthetically designed to ensure the proper and sequential placement of DNA modules [16]. One of the major aspects that contributed to propelling the GG cloning approach is its ability to build complex plasmids comprising several custom TUs through a single, one-pot reaction [17–19].

Over the years, the versatility, low cost, and high modularity of GG cloning has echoed in all major model systems (e.g., yeast, bacteria, and mammals), including plants [20]. The swift development in plant cloning systems relying on different Type IIS restriction enzymes (e.g., BsaI, BbsI, and PaqCI) has given rise to a large number of plasmid kits (e.g., MoClo Plant Kits, GoldenBraid/GB, and GreenGate), now available through Addgene and other platforms [21–23]. Although this large diversity in plasmid kits is desirable for scientists in pursuit of increasing the complexity and throughput of their experiments, it also poses compatibility issues which can limit the implementation of these systems in the cloning routine of starting labs. Generally, these compatibility issues stem from two main aspects associated specifically with GG cloning: (i) the

overhang syntax adopted by each system (e.g., PhytoBrick, GreenGate, MK/MPAT or others); and (ii) the degree of domestication (i.e., the removal of internal Type IIS recognition sites) of the DNA modules relative to the enzymes used [1].

To facilitate the adoption of GG cloning in starting labs, we review here the different GG cloning kits currently available for the plant science community, with detailed insights regarding the modules and cloning strategies found in these kits. We additionally discuss domestication strategies for noncoding and coding sequences (CDS) and highlight alternative solutions, such as methylase-assisted assembly (e.g., MetClo) [24], for modules for which domestication is not an option (e.g., modules that require perfect sequence integrity, such as those used for functional validation). Overall, this review aims to support entry-level plant synthetic biologists by providing a broad overview of the GG cloning field and a compilation of more than 850 DNA modules currently available for GG cloning.

2.0 Mechanism of Golden Gate cloning

Basic Mechanism

GG cloning relies on the use of Type IIS restriction enzymes which cleave their target outside their recognition sites — hence their common designation as "shifted endonucleases" [18,25] (Box 1). With proper design, the shifted nature of these enzymes can be harnessed to assemble a high number of modules — either smaller parts such as promoters and terminators or larger components such as TUs (i.e., a suite of modules that can be transcribed by RNA polymerase) — in a one-pot reaction that combines a specific Type IIS endonuclease (e.g., T4 or T7 DNA ligases) [18]. Mechanistically, Type IIS enzymes first bind to their recognition sites and then cleave the DNA a fixed number of nucleotides away, creating single-stranded overhangs [26]. These overhangs serve

as fusion sites: complementary sequences from different DNA fragments anneal and are covalently joined by the ligase, enabling the seamless and directional assembly of multiple modules into a functional construct [18].

One of the main advantages of Golden Gate cloning is its exceptional versatility at the design stage: by embedding Type IIS recognition sequences and custom-designed overhangs into primers (via PCR amplification or similar strategies), DNA fragments can be engineered in advance to assemble in a defined sequence and orientation (Fig. 1). The identity and placement of these overhangs determine the precise sites of cleavage and joining, providing high specificity while enabling the concurrent assembly of multiple fragments. As the recognition sites are eliminated during the reaction, the method produces scarless or nearly scarless constructs and facilitates efficient, iterative assembly within a single one-pot digestion–ligation workflow. Together, this precise coordination of cleavage and ligation forms the basis of the efficiency and modular nature of Golden Gate cloning.

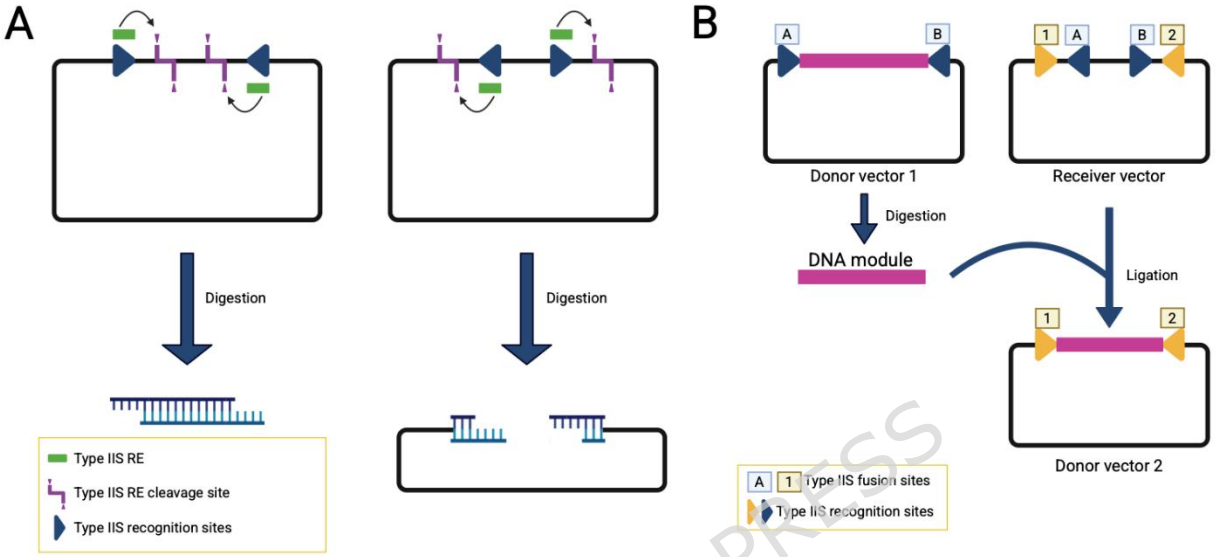

Figure 1. Different orientations of Type IIS recognition sites determine module excision and insertion. (A) Inward-oriented recognition sites are used to excise out a DNA module for further assembly (cargo is retained, while backbone is removed), while outward-oriented sites digest the vector to accept a new DNA module of interest (backbone is retained, while cargo is removed). **(B)** The receiver vector contains two pairs of Type IIS recognition sites, allowing it to function as a donor vector in the second assembly cycle. Type IIS restriction enzymes recognize these sites and cleave outside of their recognition sequences, generating defined fusion sites (A, B, 1, 2) that direct DNA assembly.

In molecular biology, Type IIS restriction enzymes typically recognize asymmetric DNA sequences that are 4 to 7 bp long and cut outside of these recognition sites, generating overhangs ranging from 0 to 5 bp [27]. Experimentally, most Type IIS restriction endonucleases used for GG cloning can be classified into two simplified classes: (i) 3-bp cutters (e.g., SapI); and (ii) 4-bp cutters (e.g., BsaI). The SnapGene software (www.snapgene.com) and New England Biolabs website respectively list 71 and 49 Type IIS restriction enzymes, with a total of 45 that are common to both databases (Supplementary table 1A). Among them, nine — two generating 3-bp sites and seven creating 4-bp sites — are widely employed across various biological systems and can be considered the core GG enzymes (Table 1). A few systems, such as GreenGate 1.0, rely a single restriction enzyme, while the vast majority use the sequential combination of two or more Type IIS restriction enzymes (e.g., Level 0/BbsI and Level I/BsaI) to enable the consecutive assembly of multiple TUs. Yet, all rely on enzymes that generate either 3-bp or 4-bp overhangs to ensure precise and efficient DNA fragment assembly [19]. Overall, these core GG enzymes collectively recognize seven distinct DNA sequence motifs: one motif from the two 3-bp–overhang enzymes and six motifs from the seven 4-bp–overhang enzymes.

Table 1. Main Golden Gate restriction enzymes and their associated isoschizomers.

Golden Gate restriction enzyme and isoschizomer	**Recognition site**	**Overhang size**
SapI* / BspQI* / LguI / PciSI	5′-GCTCTTC(N^1/N^4)-3′	3-bp
BbsI* / BpiI / BstV2I / BpuAI	5′-GAAGAC(N^2/N^6)-3′	4-bp
BfuAI* / BspMI	5′-ACCTGC(N^4/N^8)-3′	4-bp
BsaI* / Eco31I / Bso31I / BspTNI	5′-GGTCTC(N^1)/(N^5)-3′	4-bp
BsmbI* / Esp3I*	5′-CGTCTC(N^1/N^5)-3′	4-bp
BtgZI*	5′-GCGATG(N^{10}/N^{14})-3′	4-bp
PaqCI* / AarI	5′-CACCTGC(N^4/N^8)-3′	4-bp

* Indicates the main restriction enzymes designated as part of the Golden Gate framework.

Typically, most standardized GG systems have coalesced around enzymes generating 4-bp overhangs, as these allow the creation of the widest variety of fusion sites while preserving the capacity to interoperate between all 4-bp cutting enzymes [19,28,29]. While a total of 256 unique flanking sequences can be theoretically obtained with 4-bp cutting enzymes, it is generally recommended to avoid the 16 palindromic sequences, thus reducing the number of effective overhangs to 240 [18]. Additionally, overhangs differing by only a single base should be avoided, as such minimal variation can lead to mismatches [17]. A handful of systems, such as Start-Stop assembly — which employs SapI — use endonucleases that generate 3-nucleotide overhangs [20,30]. This strategy, while constrained by the limited number of possible overhangs (i.e., 64 unique trinucleotides), preserves the reading frame of assembled sequences [20,30]. However, systems based on 3-bp cutters are more limited in scope and practicality because only a single suitable recognition sequence is available, in contrast to the six distinct recognition sequences of the common 4-bp-generating Type IIS enzymes. In principle, 5-bp-cutting enzymes such as HgaI or BaeI could provide an even larger combinatorial space of fusion sites than 3-bp and 4-bp cutters (i.e., 1,024 pentanucleotides); however, they have seen little adoption in the community due to their impractical recognition sequences or cutting scheme. HgaI, for instance, recognizes the short and frequent GACGC motif, thus complicating the domestication process of sequences and limiting its usefulness for standardized assembly. Similarly, BaeI, despite having a long recognition sequence advantageous for predictable patterning, cleaves at two distinct positions, preventing the reliable generation of uniform overhangs required for GG workflows. Regardless of whether a 3- or 4-bp syntax is used (or a 5-bp scheme, should suitable enzymes become available), the general workflow of a typical Golden Gate reaction remains largely unchanged, consisting of repeated cycles (typically ≥10) alternating between digestion (e.g., 37 °C) and ligation (e.g., 16 °C), followed by a final incubation to remove residual restriction sites and a heat inactivation step [31].

Linear additive and Multiplicative Assemblies

By using the shifted cleavage properties of Type IIS restriction enzymes, researchers can easily generate new DNA modules at different positions of a transcriptional unit (e.g., promoter, CDS, or terminator) by applying the position-specific fusion sites generated by the GG restriction enzymes and defined by standards such as the PhytoBrick syntax [1]. Following the creation of new TUs, these units can then be assembled into multigene constructs. Two non-mutually exclusive GG cloning schemes — linear additive and multiplicative — are currently used to generate assemblies containing multiple TUs [24] (Box 1; Fig. 2). Both approaches rely on multiple GG restriction enzymes to assemble the TUs in a stepwise modular manner across different levels, a mode of cloning called hierarchical assembly [32].

- At Level 0, the basic DNA modules (e.g., promoters, coding sequences, and terminators) are cloned into different entry vectors using a set of standardized overhangs to form the basic parts;

- At Level 1, the basic parts are assembled using a single Type IIS restriction enzyme to form a fully functional unit that can be transcribed upon the action of RNA polymerase;

- At Level 2, a different GG restriction enzyme can be used to clone the different TUs into a single multigene construct.

However, these approaches differ in the topology used to clone single multigene construct, which is found at Level 2. In the linear additive approach, the Level 2 construct are being assembled in a predefined and unidirectional manner, where each transcriptional unit is added sequentially into a

higher-order vector (Fig. 2A). In this system, the number of TUs that can be incorporated is predefined (e.g., up to *X* TUs) and is constrained by the design of the destination vectors. The positioning of modules within the destination vector is determined by the choice of overhangs, and plasmid closure at a specific position requires the use of a chain-terminating module (e.g., modules suffixed with "Ts" in the JMC approach) [8]. In contrast to systems employing a multiplicative topology, linear additive systems are considered "locked" after the final assembly step, as no GG restriction enzyme recognition sites remain available for further cloning. The most well-known system employing this approach is Modular Cloning, also named MoClo. The original kit was introduced in 2011 by Weber et al. along with a set of basic parts and has since been expanded through multiple plant-specific compatible kits [7,8,32]. Apart from plants, MoClo kits have been developed for a wide range of organisms, bacteria such as *Escherichia coli* [33,34], filamentous fungi such as *Penicillium* or *Aspergillus* species [35,36], and mammals [37,38].

The latter approach — known as multiplicative or loop GG cloning — relies on the use of two different Type IIS restriction enzymes to combine composite parts together (Fig. 2B, 3) [39]. The looping topology is achieved through the alternating arrangement of Type IIS restriction sites, enabling the vector to be expanded indefinitely through the sequential addition of DNA modules [39]. To build vectors that allow for looping, the alternating assembly levels must all harbor recognition sites for two distinct Type IIS restriction enzymes with their sites arranged in opposite orientations (Fig. 2). As a consequence, for this specific design, the recognition sites of the non-cutting enzyme are always preserved, thus enabling the looping motion. Overall, this simple cloning design reduces the number of unique overhangs required, thereby simplifying preparation and easing the reuse of standardized parts. Unlike linear additive systems, the looping procedure is

not “locked,” as it does not rely on a fixed number of positions. As a consequence, the process can, in principle, continue indefinitely, which is why it is often referred to as “endless assembly” (Fig. 3); however, it is ultimately constrained by the size of the final construct, as excessively large plasmids tend to reduce transformation efficiency. GoldenBraid [39] is such an approach that relies exclusively on a looping procedure to perform the concatenation of TUs, while some other linear additive systems such as Joint Modular Cloning or the extended version of MoClo have integrated modules that enable looping functionalities [8,40].

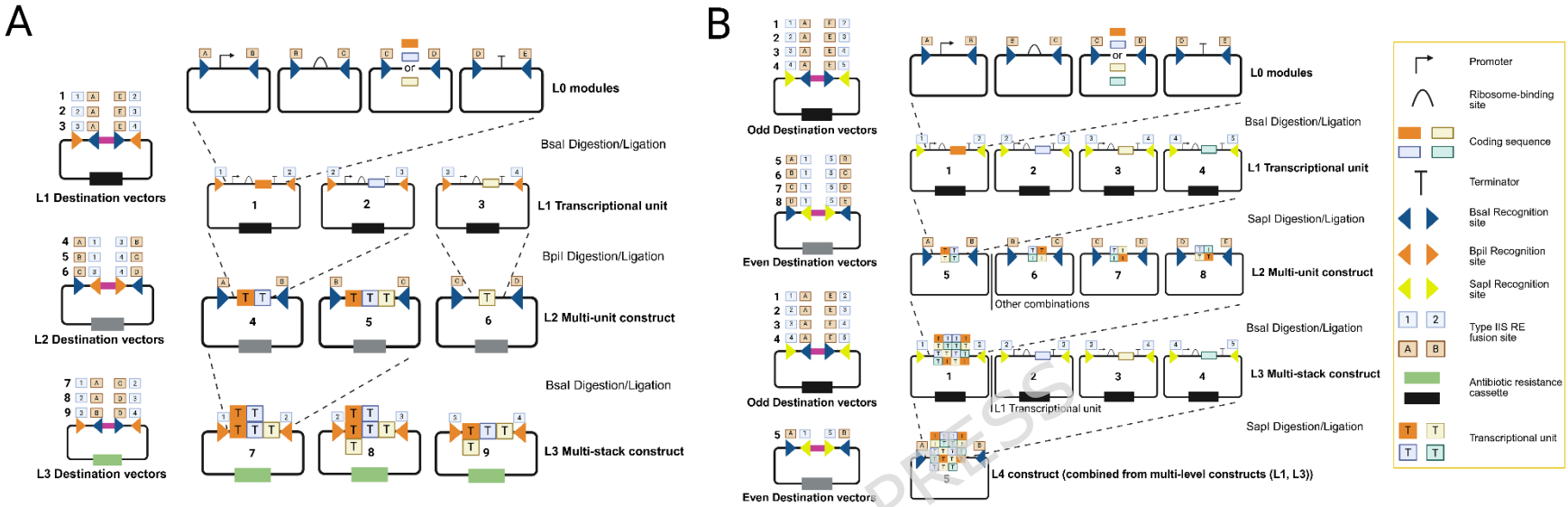

Figure 2. Comparison of linear additive and multiplicative Type IIS-mediated DNA assembly.

(A) Linear additive assembly. It proceeds through successive levels (L0–L3) by alternating between Type IIS restriction enzymes (e.g., BsaI and SapI). L0 modules are first assembled into L1 transcriptional units and further assembled into L2 multi-unit constructs and L3 multi-stack constructs. By designing complementary fusion sites between the vectors that receive the module and the next destination vector, this design enables flexible assembly of varying numbers of transcriptional units at the same level (2, 3). However, this flexibility requires each level to use distinct destination vectors that align with the intended assembly design. **(B) Multiplicative assembly.** It is represented in a hierarchical format for comparison. Similar to linear additive assembly, DNA modules are assembled through successive levels by alternating between two Type IIS restriction enzymes (e.g., BsaI and SapI). Instead of designing distinct destination vectors for each purpose, loop assembly alternates between groups of "odd" and "even" destination vectors. Loop assembly can achieve a similar

level of flexibility by assembling constructs from different levels together. As odd assembly levels such as L1 and L3 use the same destination vectors, constructs can be assembled together even if they originate from different levels, a process referred to as mixed-level assembly. For example, three L1 transcriptional unit vectors can be assembled together with an L3 multi-stack construct vector after SapI digestion/ligation to form an L4 construct shown in figure. Therefore, loop assembly can be viewed as a modified form of linear additive Golden Gate assembly that uses standardized fusion site order to enable recursive assembly. To facilitate understanding, the notation used here follows that shown in Fig. 1.

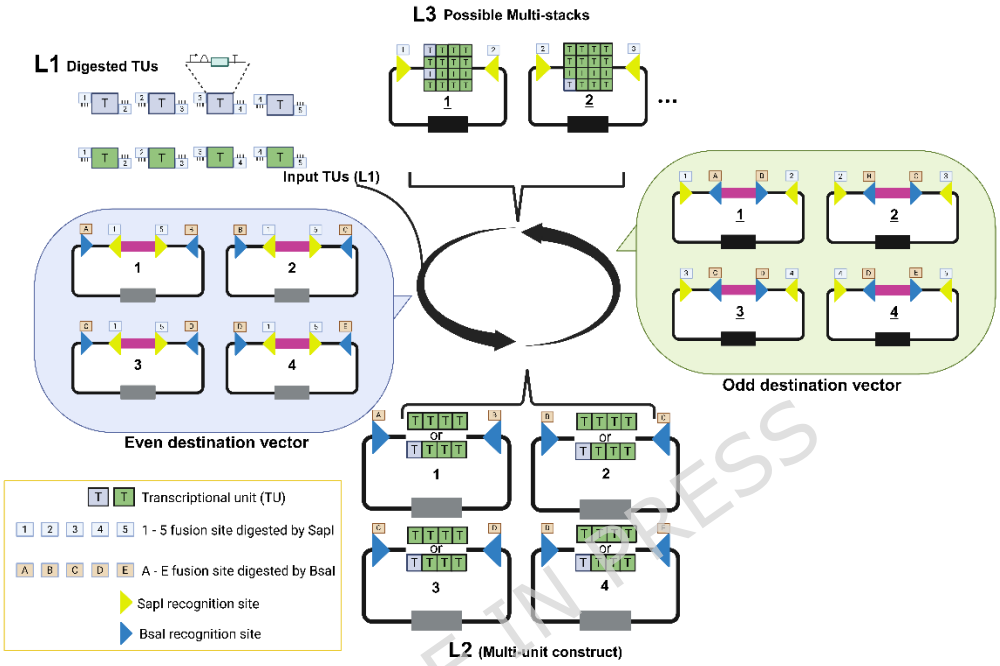

Figure 3. Cyclic representation of a multiplicative assembly based on the Loop assembly framework. DNA modules are first assembled into odd destination vectors, where SapI digestion release them as L1 transcriptional units (TUs) for further assembly. These TUs are then assembled into four types of even destination vectors to generate L2 multi-unit constructs. At the L2 stage, two possible assembly combinations (indicated by "or" in this panel) illustrate the flexibility available for generating L3 constructs. Subsequent digestion with BsaI digestion and reassembly into odd destination vectors produces a fourfold increase in assembled products with each cycle. By alternating the orientation of SapI and BsaI fusion sites (1, 2, 3, 4, 5 and A, B, C, D, E, respectively) between inward and outward configurations, cyclic (looping) assembly is achieved. Notably, the fusion sites flanking the dropout cassette are fixed, with 1 and 5 for even destination vectors and A and E for odd destination vectors. This design enables recursive and theoretically unlimited expansion of DNA constructs.

3.0 Defining the Syntax to Standardize Cloning Procedures

Design and procedure

In the early adoption of Type IIS restriction enzymes to perform GG reactions, several laboratories chose to convert previously available plasmids by assigning compatible overhangs to specific regulatory modules (e.g., promoters, coding sequences, and terminators), often with a non-interchangeable syntax [1]. In 2015, Patron et al. proposed a common set of rules based on standardized 4-bp overhangs, a system known as the PhytoBrick syntax, with the goal of establishing an extensive catalogue of standardized and well-characterized DNA parts specifically tailored for plant bioengineering, hence the name PhytoBrick. PhytoBricks were developed to overcome limitations of earlier DNA standardization syntaxes, including BioBricks™ [41,42] and subsequent systems such as the Freiburg standard [43] and BglBricks, which did not rely on Type IIS restriction enzymes [44]. While BioBricks™ introduced a hierarchical cloning strategy aimed at creating interchangeable DNA parts for the synthetic biology community [41,42], it suffered from several key limitations: (i) labor-intensive and slow assembly; (ii) lack of a modular hierarchy; (iii) formation of unwanted scars during the assembly process; and (iv) lack of versatility between systems. Consequently, the emergence of GG cloning provided an opportunity for the development of PhytoBrick as a robust, highly interoperable standard in the realm of plant cloning that effectively addressed these shortcomings. Its advantages led to its widespread adoption across numerous GG-based plant-specific cloning platforms (e.g., GoldenBraid and most MoClo variants) [19,32,39], as well as more generalist GG systems such as Möbiusand Universal Loop [45,46].

In essence, the PhytoBrick standard breaks down the TU into 10 fragments, each flanked with unique fusion sites at their 5′ and 3′ termini (Fig. 4a; Supplementary table 1B) [1]. During the assembly of these PhytoBricks, the fusion sites between fragments of DNA need to be complementary for proper binding, a required condition for the formation of a continuous functional sequence of DNA [1]. Following digestion and ligation, these fragments are assembled into a functional TU, which then can be transcribed upon binding by RNA polymerase. According to the literature, PhytoBrick modules can be defined by their module names spanning from the distal promoter (A1) to the terminator (C1) [1] and by their positional overhangs, which range from J1 (5′ junction of distal promoter) to J11 (3′ overhang of the terminator) [8]. The DIST, PROX, and CORE modules correspond to the 5′ non-transcribed region, with the transcription start site located within the CORE promoter module [1]. The transcribed region is subdivided into three (5′UTR + CDS + 3′UTR) or four (NTAG or CTAG + 5′UTR + CDS + 3′UTR) modules. To streamline the cloning workflow and maximize modular flexibility, the 5′ overhang of the PhytoBrick CDS1 module (position B3) encodes a methionine by incorporating an ATG start codon (i.e., AATG). The 3′ non-transcribed region corresponds to the terminator and contains the polyadenylation sequence. Although ten fragments are defined in the original PhytoBrick approach, some can be substituted by larger composite fragments. This procedure is generally done to ease the cloning burden and reduce the general complexity of their experiments. As such, scientists often use simplified spanning fusion sites using primarily the (i) distal promoter, cis-regulator, or transcriptional enhancer (DIST/A1), (ii) minimal promoter, including the transcription start site (CORE/A3), (iii) N-terminal coding region (NTAG/B2), (iv) C-terminal coding region (CTAG/B5), and (v) transcription terminator including the polyadenylation signal (TERM/C1) [1,8]. In that spirit, the PhytoBrick system offers extensive customization, enabling the cloning of

TUs for a variety of functions, including C-tag and N-tag fusions, protein interaction analyses, and promoter studies (Table 2)

Table 2. Different types of transcriptional units and their assembly using PhytoBrick building blocks.

Function[&]	Building blocks for specific functions[$ £]	Purpose / Notes
Basic transcriptional unit expression	*GGAG* - PROM + 5′UTR - *AATG* + *AATG* - CDS* - *GCTT* + *GCTT* - 3′UTR + TERM - *CGCT*	Standard protein expression without tags or targeting signals.
Secreted protein	*GGAG* - PROM + 5′UTR - *AATG* + *AATG* - CDS1 - *AGCC* + *AGCC* - CDS2 + CTAG* - *GCTT* + *GCTT* - 3′UTR + TERM - *CGCT*	The tag includes a secretion signal which directs the protein to the secretory pathway.
C-terminal fusion (CT-fusion)	*GGAG* - PROM + 5′UTR - *AATG* + *AATG* - CDS1 + CDS2 - *TTCG* + *TTCG* - CTAG* - *GCTT* + *GCTT* - 3′UTR + TERM - *CGCT*	Enables addition of a C-terminal tag or fusion domain.
N-terminal fusion (NT-fusion)	*GGAG* - PROM + 5′UTR(f) - *CCAT* + *CCAT* - NTAG - *AATG* + *AATG* - CDS* - *GCTT* + *GCTT* - 3′UTR + TERM - *CGCT*	Enables addition of a N-terminal tag or fusion domain.

N- and C-terminal fusion	*GGAG* - PROM + 5′UTR(f) - *CCAT* + *CCAT* - NTAG - *AATG* + *AATG* - CDS1 + CDS2 + *TTCG* + *TTCG* - CTAG* - *GCTT* + *GCTT* - 3′UTR + TERM - *CGCT*	Allows tagging or fusion at both the N- and C-termini of the protein.
Promoter variant A	*GGAG* - DIST + PROX - *TCCC* + *TCCC* - CORE + 5′UTR - *AATG* + *AATG* - CDS* - *GCTT* + *GCTT* - 3′UTR + TERM - *CGCT*	Synthetic or regulatory promoter variant with modified A-block configuration.
Promoter variant B	*GGAG* - DIST - *TGAC* + *TGAC* - PROX - *TCCC* + *TCCC* - CORE + 5′UTR - *AATG* + *AATG* - CDS* - *GCTT* + *GCTT* - 3′UTR + TERM - *CGCT*	Synthetic or regulatory promoter variant with modified B-block configuration.
Protein interaction module	*GAGG* – Adaptor motif - *AATG* + *AATG* - CDS* - *GCTT* + *GCTT* - 3′UTR + TERM - *CGCT*	Incorporates an adaptor motif for protein–protein interactions or scaffolding.

[&] This table is inspired by the multipartite PhytoBrick configurations illustrated in the GoldenBraid system (adapted from the GoldenBraid Pro multipartite figure, https://goldenbraidpro.com/do/multipartite/)

[$] Green and red colors, non-transcribed regions. Blue color, transcribed region.

[£] The * symbol indicates the presence of a stop codon.

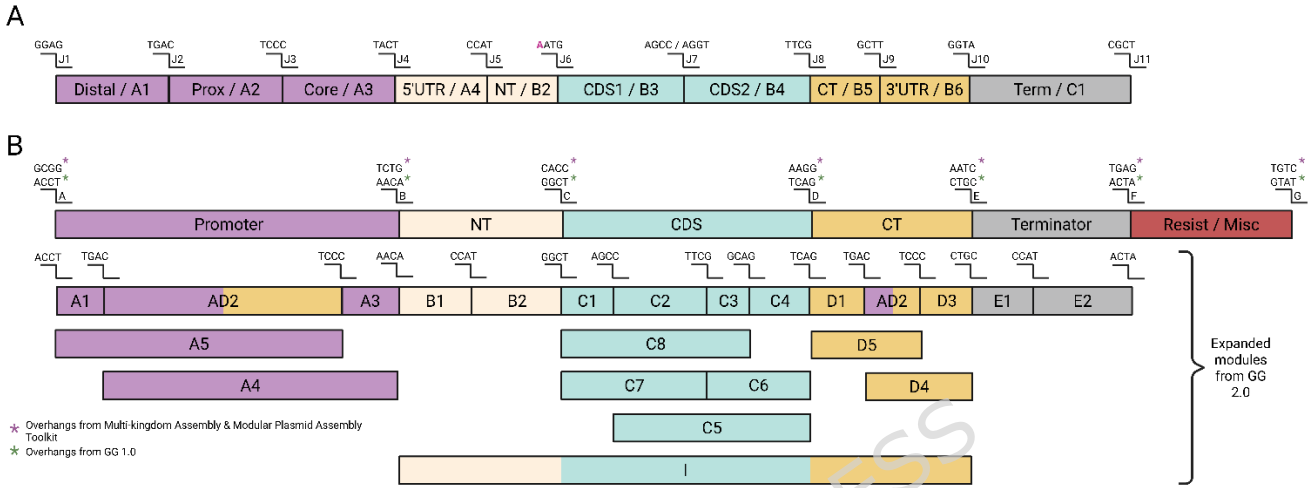

Figure 4. Common syntax used in plant Golden Gate cloning systems. (A) PhytoBricks. Modular structure showing the standard J1–J11 overhangs that define boundaries between functional regions, including distal and proximal promoter segments, core promoter, 5′UTR, non-translated region (NT), CDS1/2, C-terminal region (CT), 3′UTR, and terminator. **(B) GreenGate 1.0 & 2.0 and the Multi-Kingdom / Modular Plasmid Assembly Toolkit.** Typical structure of the GreenGate 1.0 and Multi-Kingdom systems, composed of six modules defined by seven standardized overhangs. GreenGate 2.0 introduces an expanded architecture with a broader range of modules spanning promoters, NT regions, CDS, CT elements, terminators, and resistance/miscellaneous parts. Submodules integrated in GreenGate 2.0 are designated A1–A4 for the promoter region, B1–B2 for the NT region, C1–C8 for the CDS region, D1–D4 for the CT region, and E1–E2 for the terminator region. Note that the AD2 submodule carries overhangs that allow it to function in both the promoter and CT regions and that the I submodule spans the NT, CDS and CT regions. In GreenGate 2.0, these submodules are not assigned fixed functional roles (e.g., distal or core promoter), allowing positions to

be flexibly arranged to enhance cloning versatility. This enhanced versatility can be used to build complex systems such as constructs containing multicistronic bacterial expression cassettes, multiple open reading frames or several guide RNAs.

systems such as constructs containing multicistronic bacterial expression cassettes, multiple open reading frames or several guide RNAs.

As previously mentioned, not all GG systems comply with the PhytoBrick guidelines. GreenGate, an alternative to MoClo and other PhytoBrick-complying systems, is a highly modular plant-specific cloning approach that assembles plant TUs using a different syntax based on six defined DNA modules in its most basic format (Fig. 4b; Supplementary table 1B) [47]. Unlike other systems, GreenGate uses a single Type IIS restriction enzyme (BsaI), which reduces the need for extensive domestication of internal recognition sites [47]. Specifically, GreenGate uses seven types of overhangs that are based on an "A-G" nomenclature for six different modules (promoter – N-tag – CDS – C-tag – terminator – resistance), with each module's 5′ overhang on the top strand being complementary to the 3′ overhang of the neighboring module on the bottom strand. Uniquely, this system defines a specific position (i.e., position "F-G") for the cloning of a plant selectable cassette to facilitate transgene stacking. This approach was chosen to circumvent some of the issues associated with the selection of transgenic events in secondary or tertiary transformations, such as the need to stack multiple resistance cassettes in a single transgenic line (e.g., Salk [48] or SAIL [49] T-DNA insertion mutant collections). To achieve this, the developers of the GreenGate cloning system designed a collection of eight different plant resistance cassettes accounting for five different selectable markers (i.e., resistance to glufosinate/Basta^{R}, kanamycin/Kan^{R}, hygromycin/Hyg^{R}, *sulfadiazine*/Sulf^{R}, and D-alanine/D-ala^{R}) which are cloned downstream of the terminator sequence.

GreenGate 2.0 further refines the modular architecture of GreenGate 1.0 by dividing the original modules A–E (corresponding to promoter, N-terminal tag, coding sequence, C-terminal tag, and

terminator modules, respectively) into 14 submodules and also introducing 8 composite submodules to enhance versatility (Fig. 4b; Supplementary table 1B). For instance, the A module — which represents the promoter region — can be composed of up to three submodules (A1 and/or AD2 and/or A3 and/or A4 and/or A5). Importantly, GreenGate 2.0 does not assign fixed positional roles (such as proximal or distal promoters) to these submodules, as the PhytoBrick standard does. As such, GreenGate 2.0 preserves full modularity and design flexibility within an experimenter's cloning strategy, but also limits interoperability across the community, both within GreenGate and with other modular cloning systems. Another system that has adopted a syntax similar to GreenGate is Multi-Kingdom (MK)/Modular Plasmid Assembly Toolkit (MPAT) Golden Gate. In a similar fashion, this system has introduced a "A-G" nomenclature with seven overhangs for the cloning of six different modules: (i) promoter (A-B); (ii) N-tag (B-C); (iii) gene of interest (GOI; C-D); (iv) C-tag (D-E); (v) terminator (E-F); and (vi) miscellaneous (F-G) (Fig. 4b; Supplementary table 1B). In summary, despite the existence of various other non-GG (e.g., BioBricks™) and GG–based (e.g., Start-Stop Assembly) standards within synthetic biology, the majority of plant-oriented frameworks have chosen PhytoBrick (most important), GreenGate (second in importance) and MK (lesser extent) as their primary syntaxes [50].

Development of a standardized 3-bp syntax

Although the main syntax is built around a 4-bp standard, there is growing interest within the community in developing an alternative framework based on 3-bp overhangs. One such approach, the Start–Stop assembly system, was introduced in 2019 and uses a 3-bp syntax to maintain the reading frame across junctions [30]. This design eliminates scars at sensitive coding sequence boundaries, such as the start codon, thereby minimizing the risk of unintended frameshifts. While 4-bp syntaxes have proven highly versatile and easy to design, the introduction of a 4-bp scar

between DNA parts, especially in regions immediately upstream of the start codon, can impair expression or introduce frameshifts, both of which may have severely deleterious effects on final protein structure and function [51]. In the case of a fusion protein, 4-bp overhangs produced by Type IIS enzymes generally require two additional flanking nucleotides to maintain the correct reading frame and build a functionally scarless assembly, while it is not the case for 3-bp overhangs [19]. The requirement for extra nucleotides in 4-bp overhangs can increase the risk of design errors. One way to address this is to convert existing 4-bp syntaxes into a 3-bp standard, which simplifies sequence design and reduces potential mistakes. Previous work has shown that such a conversion is theoretically feasible. For example, Pryor et al. (2020) demonstrated that up to 13 fragments can be efficiently assembled using SapI, suggesting that a 3-bp common syntax could be practical. In principle, this approach could be applied to existing frameworks such as PhytoBricks (based on a 10-position system), GreenGate (which uses a 6-position system), or, more generally, to any current 4-bp systems, as these typically involve fewer than 13 positions. One distinct advantage of SapI is that it recognizes the long motif GCTCTTC, thereby limiting domestication requirements due to its reduced frequency in DNA sequences. While the 3-bp overhang approach provides clear design benefits in maintaining reading frame integrity relative to the 4-bp standard, it remains constrained by several limitations, most notably the smaller set of available fusion sites (32 possible unique trinucleotides instead of 128 unique tetranucleotides), the restricted range of enzymes suitable for producing 3-bp overhangs, and a reduced availability of domesticated modules [19].

4.0 System Standardization and Interoperability Beyond Domestication

At its roots, the domestication of DNA sequences is achieved by removing a few (partial domestication) or all (full domestication) internal recognition sites of specific restriction enzymes (e.g., BsaI or Esp3I) from the target DNA [17] (Box 2). As a result, a given sequence can be partially domesticated for one enzyme (if some sites persist) and fully domesticated for another (if all sites for one specific enzyme have been excised). To maximize cloning efficiency and minimize errors, it is essential to select destination vectors and DNA modules that lack internal recognition sites for the Type IIS enzymes used, with the exception of the specific sites intentionally placed for ligation. This is because these enzymes cleave indiscriminately at all recognition sites, including any unintended internal ones [19]. In some cases, domestication can be circumvented by retaining internal "illegal" recognition sites and proceeding directly to higher-level assemblies. However, this approach is constrained by the limitations of the GG cloning system and requires careful design, thereby deviating from the one-tube, one-pot principle that facilitates a streamlined cloning workflow. In practice, a DNA module can be domesticated using a set of molecular techniques that can be used as stand-alone strategies or in combination: (i) site-directed mutagenesis; (ii) oligo stitching; (iii) synthetic reconstruction; (iv) phylogenetic search; and (v) PCR-amplified segments ligation [1,53]. Two additional strategies can be used to circumvent the removal of recognition sequences, either in coding sequences or regulatory elements such as promoters and terminators: (i) methylase-assisted assembly and (ii) multiple cloning sites.

Site-directed mutagenesis (domestication)

Site-directed mutagenesis is a PCR-based strategy that introduces specific nucleotide changes into a vector in order to determine the precise roles of individual amino acids in protein structure and function [54]. Most commonly, site-directed mutagenesis is performed by designing an oligonucleotide that contains an internal mismatch and is complementary to the targeted single-stranded DNA template [55]. During PCR, the primer anneals to the template, and the DNA polymerase incorporates the mismatch into the newly synthesized strand, thereby introducing the desired nucleotide change. This approach can be used to create point mutations, insertions, deletions, or silent changes for codon optimization or restriction site removal. For the domestication of DNA modules, the internal mismatch is used to generate a silent mutation that codes for the same amino acid as the non-mutated form, thus allowing for the removal of internal restriction sites without modifying the protein's functionality. Site-directed mutagenesis is particularly advantageous for large plant DNA sequences containing one or a few recognition sites, for which full-gene synthesis would be comparatively costly. A representative example of module domestication through site-directed mutagenesis is the artificial open reading frame *RUBY*, which encodes three enzymes involved in betalain biosynthesis which are linked by P2A peptide linkers [56]. In practical terms, the published RUBY construct is relatively large (3,957 bp; *CYP76AD1–P2A–DODA–P2A–glucosyltransferase*), and contains a single PaqCI recognition site located within the P2A linker bridging *CYP76AD1* and *DODA*. Given its considerable size and the presence of only one recognition site, site-directed mutagenesis could represent a more practical and cost-effective strategy than *de novo* synthesis in this case.

Oligo stitching (domestication)

A flexible strategy for sequence domestication involves assembling single-stranded DNA (ssDNA) oligonucleotide inserts with double-stranded DNA (dsDNA) vector fragments using Gibson

Assembly, a process referred to as oligo stitching or oligo bridging [57]. In this approach, the 5' ends of the linearized vector and donor fragment are chewed back by the 5' exonuclease in the NEBuilder HiFi master mix, generating single-stranded overhangs that allow the oligonucleotide to anneal to the donor fragment via a 20-bp complementary sequence. Following this step, DNA polymerase then fills in any gaps, and DNA ligase seals nicks [58]. For sequence domestication, the plasmid is first linearized at or near the target restriction site, either by PCR or enzymatic digestion, and a complementary ssDNA oligonucleotide is supplied to replace the excised sequence, effectively removing the enzyme site. In addition to sequence domestication, oligo stitching can directly assemble otherwise incompatible Golden Gate parts, create fully synthetic sequences, remove restriction sites, and facilitate plasmid-wide saturation mutagenesis. Interestingly, oligo stitching has been shown to convert overhangs both inter (between assembly standards, e.g., MoClo to GreenGate) and intra (within a standard, e.g., converting a C-terminal linker into an N-terminal linker) assembly standards [57].

Synthetic reconstruction (domestication)

The synthesis of full DNA modules, formerly constrained by prohibitive costs, has become increasingly accessible and constitutes a compelling alternative to site-directed mutagenesis, particularly in cases where multiple internal restriction sites from several different enzymes occur within a single coding sequence (GoldenBraid System 2024). On an experimental level, this option is particularly attractive because CDS can be easily codon-optimized for multiple amino acids to improve expression in the desired host organism while eliminating all illegal sites at the same time. In contrast to site-directed mutagenesis, *de novo* synthesis is particularly well suited for short coding sequences containing multiple recognition sites.

Phylogenetic search (domestication)

In cases where the number of recognition sites is prohibitive, phylogenetic searches can be used to find closely related orthologous sequences that contain fewer or an absence of internal illegal sequences but with the same codon usage for further PCR amplification (GoldenBraid System 2024). However, careful analysis is required to ensure that codon usage disparities do not introduce bias that could lead to functional divergence between the target sequence and its phylogenetically close ortholog.

PCR-amplified segments ligation (domestication)

PCR-amplified segments with compatible overhangs generated by GG restriction enzymes can be used to introduce silent mutations at precise locations. Following amplification, these segments are subsequently ligated into an entry vector to introduce the silent mutations in the original sequence. Similar to site-directed mutagenesis, this strategy is particularly useful for large modules containing only one or a few internal "illegal" sites. However, in cases of short sequences harboring multiple mutations, it might be more reliable and efficient to rely on DNA synthesis.

Online tools to aid in the domestication process

For GoldenBraid cloning, the GoldenBraid platform offers a domestication assistant that supports the design of PCR-based mutagenesis, phylogenetic searches, and synthetic reconstruction for sequence domestication (https://goldenbraidpro.com/tools/domestication/). To our knowledge, no dedicated stand-alone software exists for designing primers specifically for mutagenesis via PCR fusion. Nonetheless, the NEBridge SplitSet® Lite Golden Gate Utility (https://goldengate.neb.com) can assist in generating multiple PCR fragments with appropriate overhangs and recognition sites for subsequent assembly. This tool can also help reduce synthesis

costs by enabling the ligation of PCR-amplified fragments during synthetic reconstruction workflows. For oligo stitching, the NEBuilder Assembly Tool (https://nebuilder.neb.com/#!/) can be used to design the necessary overlaps and primers for the experiment.

Methylase-Assisted Assembly (avoidance)

Super-domestication offers a promising long-term solution to address compatibility issues in CDS between different Golden Gate systems; however, the domestication of multiple recognition sites from different enzymes can be time- and cost-intensive, ultimately limiting the throughput and scalability of the approach. Moreover, many forbidden recognition sites are located in modules outside the CDS (e.g., promoter or terminator), which limits the use of silent mutations in these regions if the integrity of these modules must be preserved. A clear example of this limitation in a non-CDS regulatory element is the constitutive double-enhanced CaMV35S promoter, which contains six BbsI sites, thereby constraining the use of this module in BbsI-dependent cloning systems [59]. To address the immediate challenges of domestication, several methylase-assisted assembly strategies, such as TNT cloning [60], PODAC [61], MetClo [24], and UniClo [62], use DNA methylation to temporarily protect internal restriction sites during cloning. To do so, methyltransferases (e.g., the CpG Methyltransferase M.SssI) are used to add methyl groups (CH_3) to specific bases within an illegal recognition site. Through the action of the methyltransferase, the site cannot be recognized or digested by the restriction enzyme which allows scientists to selectively protect restriction sites during the assembly procedure [60,61]. As such, this approach allows rapid cross-compatibility and the reuse of existing DNA modules without undergoing full domestication.

Multiple Cloning Sites as Cloning Modules (avoidance)

A straightforward approach to reduce domestication requirements involves synthesizing an array of multiple cloning sites (MCS) as cloning modules and incorporating one or multiple as full modules in the final subcloning vector (e.g., Level 2 in MoClo). Following their ligation in the final destination vector, the MCS can be digested to enable the integration of one or multiple specific modules that contain illegal recognition sites from an upstream level (e.g., Levels 0 or 1). As a whole, this approach offers numerous cloning advantages, as it enables an affordable direct accommodation of otherwise incompatible parts without the need for extensive sequence modification or re-domestication — processes that can be time-consuming or even impossible when illegal sites occur outside coding regions. Owing to its inherent flexibility, this strategy allows for the inclusion of one or multiple MCS modules, each designed to contain a limited or extensive set of restriction sites, depending on the intended cloning application. Furthermore, by strategically designing the MCS array to include rare-cutting restriction sites with 7- or 8-bp recognition sequences (e.g., NotI), this approach facilitates the integration of large modules containing multiple illegal sites associated with different Type IIS enzymes. As such, it can be used to further expand the lexicon of overhangs and streamline the cloning of challenging modules that cannot be domesticated. In essence, the use of MCS enables the concomitant use of two or more cloning approaches (e.g., MoClo, GreenGate, and Start-Stop) — specifically chosen to avoid domestication requirements for the modules of choice — for further subcloning into a final vector. Ultimately, this strategy can also function as a safeguard, permitting the reverse integration of modules into a “locked” vector — defined as a finalized construct devoid of Type IIS recognition sites — or the subsequent reintegration of functional modules such as the *ccdB* cassette. However, this approach deviates from the single, one-pot reaction approach and still requires to perform cloning procedures in another vector to assemble the TUs.

5.0 Golden Gate Cloning Systems for Plants

Over the years, several plant-specific linear additive (e.g., MoClo and GreenGate) and multiplicative (e.g., GoldenBraid and Loop assembly) GG systems relying on binary vectors have been introduced to facilitate the assembly of large, complex constructs harboring multiple TUs. The following section highlights the most widely adopted systems, with insights and details regarding their key features (structure, compatibility, and usability) in plant synthetic biology. Despite their methodological differences, these systems share common design principles that enhance their efficiency for plant cloning. In particular, the final subcloning plasmids included in these toolkits incorporate features that support efficient replication in *Agrobacterium tumefaciens*. These include left and right border sequences (LB/RB), which are essential for T-DNA transfer into plant cells. Additionally, the plasmids typically carry an origin of replication (ori) (e.g., pVS1) together with the associated replication machinery, ensuring stable maintenance and propagation in *A. tumefaciens*. This design reflects the continued preference for *A. tumefaciens*–mediated transformation, owing to its ease of use, cost-effectiveness, low copy number, and high efficiency in DNA delivery. In rare cases (e.g., pMIN-0 in the Joint Modular Cloning kit or minimal backbones with only a pMB1 ori for protoplast transformation in the Organelle marker visualization kit), vectors specifically engineered as minimal backbones for physical DNA delivery methods (e.g., biolistic or polyethylene glycol) are available for researchers employing these transformation approaches [8,63].

To streamline the review, we deliberately excluded cloning kits that are not based on a modular approach — defined here as systems in which individual genetic elements can be independently combined or swapped to generate new constructs. The literature includes numerous Golden Gate–based cloning systems developed for gRNA module assembly with various genome editing

reagents (e.g., [64–69]); however, for clarity and focus, we limit our review to cloning kits that either accommodate a wide range of modules (e.g., promoters, coding sequences, terminators) or can be integrated into broader modular cloning frameworks, such as MoClo and GoldenBraid. For instance, the CRISPR/Cas9 Toolbox from Lowder et al. (2015) employs the pYPQ plasmid suite to enable efficient assembly of functional CRISPR/Cas9 constructs in both monocots and dicots. While this toolbox is widely adopted in the community and highly effective for genome editing applications, it is largely restricted to this purpose and is not easily adapted to broader modular cloning workflows; therefore, it and similar kits were not featured in this review. Altogether, our survey identified more than 25 cloning kits across Groups 1 and 2, of which 13 provide a broad repertoire of DNA modules, collectively encompassing over 850 elements (Supplementary Table 1C). Together, these resources offer a comprehensive overview of the tools currently available to the research community.

Modular Cloning Basic Kit and Extensions (MoClo Toolkit/Basic Kit/Ext)

The MoClo Toolkit — commonly referred to as the "Basic kit" for modular cloning — is a hierarchical modular cloning system that can assemble eukaryotic multigene constructs at high efficiency [32]. In many cases, the "Basic kit" serves as the foundational building block for numerous derivative kits that have been developed more recently, and which rely on the "MoClo" strategy. The kit consists of seven Level 2 destination vectors with Kan^R, whose overhangs are compatible with each of the seven Level 1 destination vector positions. Five module types (promoters, 5′ untranslated regions, signal peptides, CDS, and terminators) are held in Level 0 destination vectors that confer spectinomycin resistance (Sp^R) and are directionally assembled into a TU at Level 1. In MoClo, the specific design of the overhangs allows for the linear addition of up to six TUs in a single step.

Both Level 0 and Level 2 destination vectors encode selectable markers: the *lacZα* gene for blue/white selection and a red color marker (*CRed*) for visual selection in Level 2, respectively. Level 0 vectors are flanked by BsaI and BpiI sites that are positioned inversely, where the Type IIS enzyme BsaI is used to release and assemble modules into a Level 1 destination vector harboring an ampicillin resistance (Amp^R) cassette for efficient counter-selection. BpiI is then used to assemble the TUs into the Level 2 destination vectors, which are Kan^R and have their *CRed* marker flanked by two BpiI sites. To enable further hierarchical assembly beyond this limit, two additional end linkers, pELB-n (for Level 2i-1) and pELR-n (for Level 2i-2) are used instead of the basic end linker (pELE-n). These use BsaI and Esp3I, respectively, to enable recursive assembly between Level 1 and Level 2 vectors. To depict the efficiency of the "Basic kit", Weber et al. (2011) successfully built a 33 kb multigene construct made of 11 TUs and 44 basic modules in three cloning steps. In 2012, further extension to the "Basic kit" introduced the vectors allowing for looping features and module concatenation, with the introduction of the P and M (where M stands for Multiplication) levels [40]. In Werner et al. (2012), Level M is employed to preassemble multiple TUs, which are subsequently subcloned into Level 2 constructs using a set of seven destination vectors and seven end-linkers; after cloning, all of these are flanked by two BsaI sites. In MoClo, Level 2i (where i stands for Intermediate) constructs have two type IIS restriction sites positioned after the final cloned transcription unit, allowing additional units to be added but preventing the subcloning of the assembled transcription units into a new vector backbone. To overcome this limitation, a new cloning level, Level P, was introduced, in which assembled transcription units are once again flanked by two type IIS restriction sites. To do so, Level P constructs need an additional set of destination vectors and end-linkers.

Numerous module kits providing collections of domesticated DNA modules based on the MoClo system have been developed over the years: (i) MoClo Plant Parts I-III (Moclo PPI, 96 Level 0 parts; MoClo PPII, 80 Level 0 parts; and MoClo PPIII, 94 Level 0 parts), (iii) MoClo CRISPR/Cas Toolkit for Plants (77 Level 0 parts and 26 Level 1 parts), (iv) Modular Biomolecular Fluorescence Complementation (23 Level 0 parts), (v) Modular Cloning Toolbox for Chloroplast Metabolic Engineering (115 Level 0 parts), (vi) Organelle marker visualization kit (9 new sets of organelle markers), and (vii) zCas9i Cloning Kit (Box 3; Supplementary table 1C). Together, these additions make MoClo one of the most comprehensive modular cloning systems currently available. Note that the zCas9i Cloning Kit provides a first set of vectors and parts that are intended for cloning with the MoClo approach, while a second is compatible with the pDGE Dicot Genome Kit [12,70].

GreenGate-Based Systems: GG1.0, GG2.0, and MultiGreen

The GreenGate (GG1.0) system is a modular cloning platform developed to efficiently and rapidly assemble plant transformation constructs [47]. Relying on a single Type IIS restriction enzyme (BsaI), GreenGate minimizes domestication requirements while assembling a transcriptional unit (TU) from six predefined modules (promoter, N-terminal tag, CDS, C-terminal tag, plant terminator, and plant resistance cassette), each supplied on its own pUC19-based entry vector. Unlike systems that adopt the PhytoBrick standard, GreenGate uses an independent "A–G" module nomenclature. Although multiple TUs can be assembled, GG1.0 provides limited scalability for constructing complex multigene assemblies compared to systems such as MoClo or GoldenBraid.

GreenGate 2.0 (GG2.0) expands the capabilities of the original system by increasing modularity, scalability, and user-friendliness [71]. GG2.0 subdivides the original six-module TU into 14

submodules, enabling a single Level 1 vector to accommodate up to 12 Level 0 modules. A Universal Entry Generator plasmid (pUEG) streamlines the creation of Level 0 modules, simplifying domestication workflows. Importantly, GG2.0 introduces an iterative assembly strategy known as GreenBraid, which allows the progressive stacking of TUs into Level 2 vectors for multigene constructs. Additional enhancements include preassembled Level 0 modules for multiplex CRISPR/Cas9 gRNA expression. GG2.0 remains backward-compatible with existing GG1.0 entry modules, facilitating integration with previous GreenGate resources.

Building upon the GreenGate architecture, MultiGreen (versions 1.0 and 2.0) extends the system to enable multiplexed gene expression both in series and in parallel [72]. MultiGreen maintains the six-module "A–G" syntax of GreenGate while introducing two additional elements for serial multiplexing: a transcription blocker module (i.e., a genetic module inserted between transcriptional units to reduce transcriptional interference between adjacent gene cassettes) with F–H overhangs and a multiplexer module with H–G overhangs. These modules support sequential stacking of TUs, with the multiplexer housing a visual reporter for rapid screening and flanked by internal Esp3I sites, thus necessitating domestication of internal Esp3I recognition sequences. Parallel multiplexing is achieved through binary intermediate vectors that allow independent expression of multiple TUs within a single transformation event. The MultiGreen toolkit comprises 48 plasmids, including 12 intermediate destination vectors and 3 final destination vectors offering Kan^R or Sp^R selection.

Multi-Kingdom Golden Gate (MK) and Modular Plasmid Assembly Toolkit (MPAT)

The MK modular cloning system is a flexible and versatile toolkit based on hierarchical assembly, developed for use in model species across different kingdoms [73]. The kit contains six

independent Level 2 plasmid backbone series, each consisting of five plasmids integrated with a range of bacterial origins of replication (RK2, pBBR1, ColE1, and RSF1010) or adapted yeast backbones for various experimental purposes. The system adopts a similar syntax to GreenGate, with unique overhangs defining the modules in fixed positions A–G (promoter, N-terminal protein fusion, CDS, C-terminal protein fusion, terminator, and additional parts). Newly made DNA parts first undergo PCR to add BpiI and BsaI restriction sites, allowing them to be cloned into the universal backbone p641-BpiI acceptor for BpiI cut-ligation, or alternatively into p641-Esp3I for Esp3I-based cloning. The Level 1 parts are then assembled into Level 2 and 3 backbones via BsaI and BpiI cut-ligation, respectively. To select for accurately assembled plasmids, the system uses a *ccdB* cassette and distinct antibiotic resistance markers for different levels (Level 1 with Sp^R ; Level 2 with Kan^R). Additionally, up to five TUs can be assembled into Level 3 backbones which contain Kan^R and *ccdB* negative selection cassettes. To reduce domestication requirements, the system incorporates *lacZ* or dummy (DMY) landing pad cassettes. By using these dummies, DNA modules that still contain BsaI or BpiI sites can be directly cloned into higher-level assemblies like Level 2 or 3. To enhance the versatility of the MK system, Chiasson et al. (2019) developed a Gateway-compatible entry vector (pENTR-BsaI-Tet) along with modules for constructing destination backbones using Golden Gate cloning. This backbone was specifically designed for subcloning L1 parts via BsaI cut-ligation to generate Gateway entry clones, which can then be transferred into an existing Gateway destination plasmid through standard LR Clonase–mediated recombination.

Binder et al. (2014) established a Modular Plasmid Assembly Kit that constructs binary plasmids for multigene expression, while also having the flexibility to be used for gene silencing and silencing rescue. The binary vectors and modules used in the kit are domesticated for BsaI, BpiI,

and Esp3I sites and contain *ccdB* for negative selection. The assembly kit includes modules in Level 1 (Gentamicin resistance/Gen^R), which are assembled into Level 2 (Amp^R and Sp^R), and then into Level 3 (Kan^R), followed by higher binary assemblies that alternate between Level 2 and 3 constructs using successive BsaI and BpiI cut-ligations. Up to 15 genes can be assembled into a Level 4 construct, and GOIs can be shared from Gateway technology since the kit's 4-bp overhangs (CACC and AAGG) are compatible.

Möbius Assembly for Plant Systems (MAPS)

Möbius Assembly is a two-level hierarchical system that achieves both simplicity and high cloning capacity for theoretically unlimited expansion through looping, while reducing domestication requirements by using the rare cutter AarI, for which PaqCI serves as an isoschizomer [45]. AarI reduces domestication needs as it is a rare cutter that recognizes 7-bp sequence, which is less common in DNA parts. The compact framework consists of one Möbius Universal Acceptor Vector (mUAV), four Level 1 Acceptor Vectors, four Level 2 Acceptor Vectors, and seven Auxiliary Plasmids. Chromogenic proteins like amilCP (in mUAV), spisPink (Level 1), and sfGFP (Level 2) are used as dropout cassettes for visible negative selection, simplifying clone screening. BsaI is used to digest Level 0 vectors for assembly into Level 1, and AarI is used to digest and assemble TUs into Level 2 in quadruples. Möbius Assembly for Plant Systems (MAPS) is a new PhytoBrick-standardized toolkit for plant synthetic biology based on a compact binary vector, pMAPS [75]. MAPS follows the structure of the original Möbius system, with a Möbius Universal Acceptor Vector (mUAV), seven auxiliary plasmids, and four variations (A–Δ) of each Level 1 and 2 pMAP destination vector to achieve a looping hierarchical quadruple system. The mUAV, auxiliary plasmids, Level 1 destination vectors, and Level 2 destination vectors carry resistance to chloramphenicol (Cm^R), Kan^R, and Sp^R , respectively. In MAPS, two enzymes, AarI (Level 0, 2)

and BsaI (Level 1), are used to release inserts. The chromogenic marker spisPINK was replaced by mScarlet-I from the earlier Möbius, resulting in the markers being amilCP (Level 0), mScarlet-I (Level 1), and sfGFP (Level 2). Additionally, pMAP vectors (3.6 kb in size) contain a fusion origin of replication (pWKS1 and pUC19 Ori) and two Left Border (LB) sequences, allowing them to replicate efficiently in both *E. coli* and *Rhizobium spp.* and backbone transfer to the plant genome. Furthermore, to prevent plasmid dimerization, a 240-bp *cer* site was added to the vector backbone. Finally, the kit introduces new Level 0 modules (bioluminescence and fluorescent protein reporters, promoters, and terminators), along with the "MethyAble" feature that allows Level 0 modules to be introduced into established constructs.

GoldenBraid-based systems (GB1.0, GB2.0, GB3.0, GB4.0, GBext, and the GB-gRNA/Cas9 toolbox)

GoldenBraid is a modular assembly system that uses a double loop ("braid") cloning design to assemble multipartite constructs in a binary manner for plant synthetic biology [39]. The system is based on two types of destination vectors, level α (pDGB_A12C and pDGB_C12B) and level Ω (pDGB_1AB3 and pDGB_3AB2), which have BsaI and BsmBI orientations reversed. The 1, 2, 3 and A, B, C in the vector names correspond to the orientation of the 4-bp overhangs generated by BsaI (1, 2, 3) and BsmBI (A, B, C). As a result, the components of the GB system are domesticated for these two enzymes. A TU, composed of DNA parts (promoter, CDS, and terminator) that are not fixed, is first assembled in a multipartite manner and then followed by four defined assembly rules that enable binary assembly in a highly idempotent way. The constructs are then assembled by alternating between the two types of vectors in iterative steps, allowing for indefinite construct growth. Using this system, Sarrion-Perdigones et al. (2011) successfully assembled 14.3 kb constructs from 19 basic parts.

GoldenBraid 2.0 (GB2.0), containing 93 plasmids, builds upon the original GB system by providing a common assembly standard of 11 defined part classes (such as promoters, CDS, and terminators) that reduce scars while keeping the binary assembly strategy for modular DNA construction [76]. For standardized construction, a universal entry plasmid (pUPD) was introduced by adding specific recognition sites to each DNA part rather than the plasmid, thus allowing for the flexible assembly of TUs. Additionally, GB2.0 uses three Type IIS restriction enzymes (BsaI, BsmBI and BtgZI), and standardizes the 4-bp overhangs (A=1, B=2, C=3) across the two types of destination vectors (α and Ω), making it easier to switch between different levels during iterative assembly. The DNA parts in GB2.0 are also compatible with GB3.0, meaning the same parts can be reused in even more advanced assembly workflows. Finally, the addition of new DNA modules and software tools like GBcloning has made the system more automated, easier to design with, and overall, more user-friendly. Following GB2.0, the GB3.0 and GB4.0 versions were also released as further evolutions of the system. In GB3.0 [77], the assembly platform adopted the PhytoBrick syntax and introduced enhanced webtool functionalities to facilitate *in silico* assembly, including the ability to track the assembly history of each composite part and maintain experimental data in the form of datasheets. At its essence, GB3.0 aimed to refine the GB assembly tools and rules through a renovated website that comprises four major sections, namely Design, Collection, Experiment and Genome Engineering. Overall, the GB3.0 repository incorporated circa 800 public physical phytobricks and >14,000 user-exclusive virtual gene elements. Before the genome editing tools introduced in GB4.0, the GB-gRNA/Cas9 toolbox established the foundational framework for assembling gRNAs and performing Cas9-based editing within the GoldenBraid system [78]. Notably, it enabled the adaptation of genetic components necessary for CRISPR-based editing and transcriptional regulation in this system. In the GB-gRNA/Cas9 pipeline, targets are first adapted

to the GoldenBraid standard using the “GB-CRISPR domesticator” and these Level 0 modules are then assembled with other standard GB parts via the “GB-CRISPR assembler” to generate guide RNA expression cassettes. Following this step, the cassettes are combined with each other and/or with a Cas9 transcriptional unit using the “GB-binary assembler”.

In GB4.0 [79], the system introduced a comprehensive expansion of software-assisted cloning, along with new Type IIS-based tools for assembling up to six tandemly arrayed gRNAs for both Cas9 and Cas12a, using gRNA–tRNA-spaced and crRNA-unspaced strategies. Using a two-step software-assisted procedure, GB4.0 introduces of a general cloning pipeline for single and multiplexed gRNAs either for Cas9 or Cas12a editing. In the first step, a spacer sequence chosen by the user is provided as input to the Single Cas9_gRNA domesticator (tS9D) or Single Cas12a_crRNA domesticator (tS12D) tools. In the second step, the Single Cas9_gRNA Assembler (tS9A) or Single Cas12a_crRNA assembler (tS12A) tools combine the outputs with the Level 0 elements such as a Pol III promoter and a Cas9 scaffold or Pol III promoter, crRNA direct repeats, and the hepatitis delta virus ribozyme. With its workflow, GB4.0 allows for the rapid construction of up to six tandemly arranged gRNAs compatible with both Cas9 and Cas12a. The GBext is an extended set of GB-compatible vectors that allow the fast assembly of multigenic constructs developed by Dusek et al. (2020). The GBext kit introduces a set of novel GB3.0 α-level plasmids that expand the GoldenBraid system’s capacity to rapidly assemble more complex genetic constructs, while remaining fully compatible with existing GB parts, most MoClo parts, and all GB modules. In GBext, four new plasmids named α11 to α14 replace the α1 plasmid of GB3.0, while retaining α2 and all Ω plasmids for compatibility and shuffling. Overall, GoldenBraid offers a platform for vector design and Level 0 parts through their website, https://goldenbraidpro.com/,

providing users with an integrated environment for modular DNA assembly, standardized part exchange, and streamlined construction of complex genetic circuits.

Modular Cloning Toolbox for Generation of Chloroplast Transformation Vectors

Vafaee et al. (2014) repurposed the GB2.0 kit assembly system for plastid transformation. The ease of incorporating newly designed flanking regions into the universal domesticator plasmid (pUPD) allows for quick adaptation of DNA parts for homologous recombination. Since the homologous recombination system remains active in plastids like chloroplasts, genetic engineering can be performed efficiently. Additionally, the ability to easily add flanking regions allows for better adjustment of DNA parts for sharing between different systems and species. Most importantly, plastids typically confer Sp^R via the *aadA* gene, regulated by the *rrn* promoter. Therefore, the resistance marker for the Ω vectors was changed from *aadA* (Sp^R) to the chloramphenicol acetyltransferase gene **(***cat***;** chloramphenicol resistance/Cm^R**)**. The kit uses the original α vector and the newly modified Ω vector (temporarily named pDGB3_Ω) for plastid transformation.

Loop Assembly

Loop Assembly is built on a recursive cloning framework that alternates between two Type IIS restriction enzymes, BsaI and SapI, to enable successive rounds of assembly into higher-level vectors [82]. In this system, Level 0 parts are assembled into a Level 1 TU within the pOdd vector (Kan^R) using BsaI. Following this, up to four Level 1 units are combined into the pEven vector (Sp^R) at Level 2 using SapI. Each subsequent round of assembly can combine an additional four plasmids into the subcloning vector, thus resulting in an exponential increase of the cloned TUs. In Loop Assembly, the overhangs generated by the BsaI digestion comply to the PhytoBrick syntax. Building on this plant-focused framework, the Universal Loop (ULoop) assembly approach was

subsequently developed to enable cross-kingdom cloning while maintaining an architecture comparable to that of its plant-specific predecessor [46].

6.0 Concluding remarks

Since its inception, GG cloning has evolved into a sophisticated network of cloning kits that utilize either iterative linear assembly, multiplicative module addition, or a combination of both. This review aims to provide a clear overview of the current options available to beginners in plant synthetic biology, with a particular focus on kits containing binary vectors as *A. tumefaciens* remains the preferred method for transformation in the community. To provide this overview, we systematically compared the structure, features, and compatibility of more than 25 plant-specific GG toolkits and compiled more than 850 DNA modules available through these numerous kits. With this staggering number of options, "the abundance of modular cloning toolkits, while addressing specific requirements, introduces complexity in toolkit selection" [20]. To solve this complexity problem, we present partial but practical solutions — including the several strategies for sequence domestication, multiple cloning sites, and methylase-based assembly — to help researchers navigate the conundrum of module standardization and system compatibility. As such, our aim is to offer useful starting points for reflection and practical anchors to help navigate the ever-expanding catalog of GG techniques, while contributing to a more comprehensive and unified framework for the community. As a final word, the emergence of high-throughput transformation methods, such as the *in planta* framework [83], opens new frontiers for plant synthetic biology (e.g., the rise of new model organisms). As such, the establishment of standardized GG cloning practices will be crucial to ensuring that these advances are broadly applicable, flexible, and

scalable across different research contexts — whether in emerging model species, focused studies (e.g., promoter characterization), or large-scale applications like crop engineering.

7.0 Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data sharing is not applicable to this article as no new datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SH and JGB conceived the review and systematically reviewed previous literature. JGB and BP provided training and mentorship. SH and JGB curated the figures. SH and JGB wrote the final manuscript. BP directed the project and provided funding.

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