ACS SyntheticBiology

Technical Note

pubs.acs.org/synthbio

A Golden Gate Modular Cloning Toolbox for Plants

Carola Engler,[†,∥] Mark Youles,[‡] Ramona Gruetzner,[§] Tim-Martin Ehnert,[†,∥] Stefan Werner,[†,∥] Jonathan D. G. Jones,[‡] Nicola J. Patron,*[,‡] and Sylvestre Marillonnet*[,§]

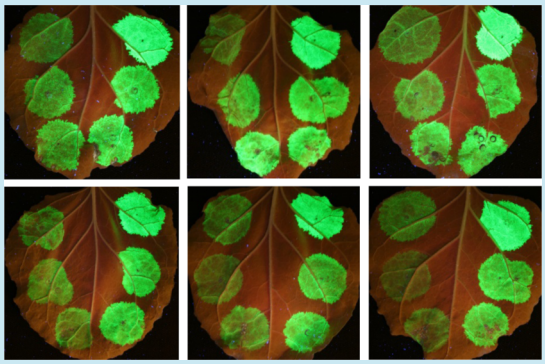
[†]Icon Genetics GmbH, Weinbergweg 22, Biozentrum Halle, 06120 Halle, Germany

[‡]The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, United Kingdom

[§]Leibniz-Institut für Pflanzenbiochemie, Weinberg 3, 06120 Halle (Saale), Germany

Supporting Information

ABSTRACT: Plant Synthetic Biology requires robust and efficient methods for assembling multigene constructs. Golden Gate cloning provides a precision module-based cloning technique for facile assembly of multiple genes in one construct. We present here a versatile resource for plant biologists comprising a set of cloning vectors and 96 standardized parts to enable Golden Gate construction of multigene constructs for plant transformation. Parts include promoters, untranslated sequences, reporters, antigenic tags, localization signals, selectable markers, and terminators. The comparative performance of parts in the model plant *Nicotiana benthamiana* is discussed.



KEYWORDS: *Agrobacterium tumefaciens, gene assembly, genetic engineering, molecular cloning, plant biotechnology, vector construction*

Inspired by engineering and mechanical assembly lines, synthetic biology relies on standardized parts to assemble genetic constructs, joining the parts together following rules known as 'assembly standards'.[1−6] The fewer the number of rules that define the standard, the more widely that standard will be adopted as the toolbox will be smaller and cheaper, and assembly will be faster and easier to automate. At the same time, versatility should not be compromised. For the assembly of genetic systems, it is necessary that standards have sufficient complexity to allow the use of the various elements of standard genetic grammar and also provide the flexibility to allow users to incorporate tags for protein purification or subcellular localization, or to fuse sequences for applications such as the study of protein/protein interactions.

Golden Gate cloning, a DNA assembly method based on the use of Type IIS restriction enzymes,[7] has many features that are optimal for development of an assembly standard. As a DNA assembly method, it avoids some of the inefficient, time-consuming, and expensive steps of molecular cloning such as PCR amplification, gel purification, and the design and ordering of custom primers. The only new expenditure for a lab already equipped for molecular cloning is to purchase specific type IIs restriction enzymes. Unlike other multipartite DNA assembly methods,[8] it does not require overlapping flanking sequences or recombination sites but only requires relatively small four base pair junctions that we call fusion sites to join adjacent modules, and even these can be designed to allow 'scarless' assembly where necessary.[9] Transcriptional units can be assembled from standard modular parts using a one-step Golden Gate cloning reaction, and higher order assembly of multigene constructs can be performed using a similar procedure.[9−11]

A limitation of Golden Gate cloning is that internal instances of the recognition sequences for the type IIs enzymes used for assembly have to be removed from all starting modules by a process known as domestication. To avoid this work being repeated in independent laboratories, access to a large toolbox of cloned and domesticated sequences would be beneficial to researchers. Such repositories have been successful for the BioBrick assembly standard.[3,4] We describe here a kit of 96 domesticated standard parts that include plant promoters and untranslated sequences, reporters, antigenic tags, localization signals, selectable markers, and terminators. This "Golden Gate Modular Cloning (MoClo) Plant Parts Kit" has been placed in the AddGene repository together with a "Golden Gate MoClo Plant Tool Kit", which contains all the vector backbones and sequences required for domestication of new sequences and assembly into single and multigene binary constructs.

RESULTS AND DISCUSSION

Golden Gate Assembly Standard for Plant Biology. The basic genetic grammar of promoter, coding sequence, and terminator has been broken down into standard parts. At this level, each part is known as a level zero module (Figure 1A and B). To conform to the assembly standard previously described

Received: September 30, 2013
Published: February 5, 2014

   dx.doi.org/10.1021/sb4001504 | *ACS Synth. Biol.* 2014, 3, 839−843

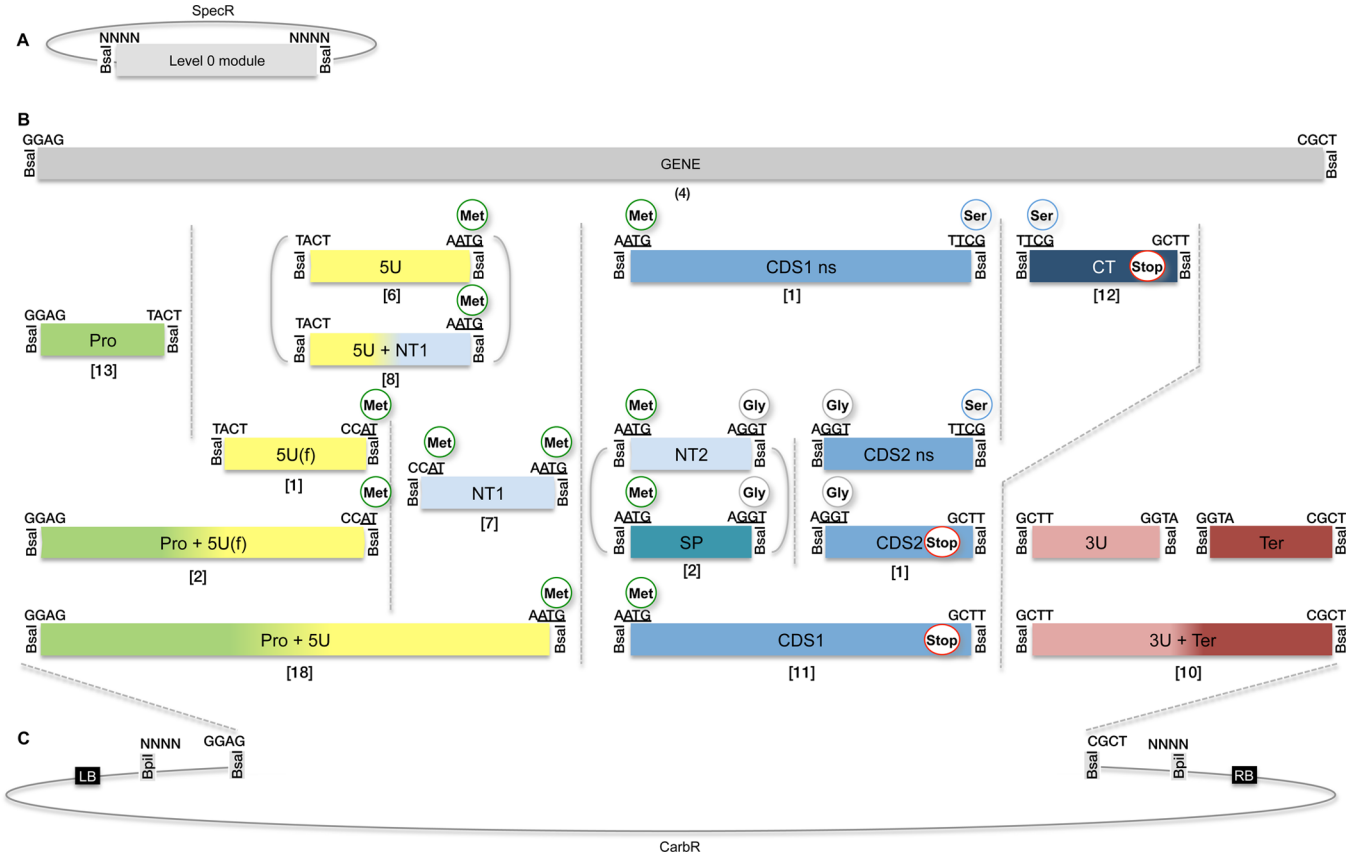


Figure 1. (A) Level zero modules are cloned in a vector backbone with a spectinomycin resistance gene and are flanked by two *BsaI* sites in opposing orientations (sequences ggtctcannnn and nnnntgagacc for the 5′ and 3 sites, respectively). (B) All parts of the same type are flanked by the same pair of fusion sites (for each fusion site, only the sequence of the top strand is shown). Numbers in square brackets below each module type indicate the number of modules supplied in the Plant Parts Kit. Modules in parentheses are of the same type but contain sequences of different nature. (C) Modules can be combinatorially assembled into a level one acceptor backbone to make transcriptional units. All level one and higher acceptor backbones have left and right borders (LB and RB) for T-strand transfer into plant cells and contain an origin of replication for *Agrobacterium tumefaciens*. Pro = promoter, 5U = 5′ untranslated sequence, 5U(f) = 5′ untranslated sequence for N terminal fusions, NT = N terminal tag or localization signal, SP= signal peptide, CDS=coding sequence, CT = C terminal tag or localization signal, 3U = 3′ untranslated sequence, Ter = terminator, ns = no stop codon.

as the Golden Gate MoClo system,[7,11,12] all level zero parts were made to meet the following requirements: (1) they are flanked by two *BsaI* sites in opposing orientations, (2) all parts of the same type are flanked by the same pair of fusion sites (Figure 1B), (3) recognition sequences for the restriction endonucleases *BsaI*, *BpiI*, and *BsmBI* are absent from internal sequences, and (4) the vector backbone contains a spectinomycin resistance gene. We have previously described a number of level zero module types including promoters (Pro), 5′ untranslated sequences (5U), coding sequences (CDS1), 3′ untranslated sequences (3U), and transcription terminators (Ter). For secreted proteins, the coding sequence module could be subdivided in two modules consisting of a signal peptide (SP) and coding sequence (CDS2). Composite modules consisting of a combination of several basic module types could also be used; for example, module Pro+5U contains both promoter and 5′ untranslated sequences.[9] Here, we define several additional module types that allow users to make N- and C-terminal fusions (modules NT1 and CT, respectively) (Figure 1). Because the left fusion site of module NT1 consists of sequence CCAT, which did not fit any of the other existing modules, we created a new module, 5U(f), for use between Pro and NT1 modules. Alternatively, a composite module containing parts Pro and 5U(f) can also be fused to module NT1. An N-terminal fusion tag can also be included in the previously defined 5U module, resulting in a module containing both 5′ untranslated and coding sequences. Finally, N-terminal fusion tags can also be cloned in the previously defined SP module; therefore, this module type can also be referred to as NT2. For C-terminal fusions, two new modules types (CDS1 ns and CDS2 ns) were required for coding sequences lacking a stop codon. The fusion site selected for joining coding sequences and C-terminal tags (sequence T TCG) was chosen to leave only one or two small amino acids (Ser or Gly-Ser) at the cloning junction (an example is shown in Supporting Information, Data 1). Combining selected sets of modules from this library will allow users to express a coding sequence in any desired combination, that is, either with or without a N- or a C-terminal fusion tag, or even with both N- and C-terminal fusion tags.

Selected level zero parts are assembled into level one acceptor vectors in a single cloning step, resulting in level one constructs containing assembled transcriptional units (Figure 1). Multigene constructs are then assembled from level one constructs in acceptor backbones referred to as level 2, M, or P acceptors (Figure 2), as previously described.[11] All level one, two, M, and P backbones have left and right borders and an origin of replication that functions in *Agrobacterium tumefaciens*.

dx.doi.org/10.1021/sb4001504 | *ACS Synth. Biol.* 2014, 3, 839−843

Figure 2. Golden Gate MoClo Assembly Standard. Standard (level zero) parts are assembled from single or multiple sequences either directly or via intermediate level −1 fragments. Level zero parts are assembled into level one acceptor backbones to make complete transcriptional units. Multigene constructs can be made by assembling level one constructs in level 2, M, or P acceptor backbones.

The Golden Gate MoClo Plant Tool Kit. Cloning level zero modules and concurrent removal of internal type IIS restriction sites requires dedicated cloning vectors. We have previously described a set of level zero cloning vectors[9] and have now updated this collection of vectors to allow cloning of the novel module types described above (Supporting Information, Data 1). In addition, we describe a universal level −1 cloning vector that facilitates the cloning of large level zero modules and the removal of internal type IIS restriction sites. This vector allows sequences of interest to be cloned in several distinct fragments as intermediate constructs. The resultant level −1 constructs are sequenced using vector primers and are then seamlessly assembled in a level zero acceptor to make the complete level zero module. A detailed description of how to construct level zero modules is provided in Supporting Information, Data 1.

All the vector backbones required for the domestication of new sequences and assembly of level zero modules into single and multigene binary constructs are provided in the Golden Gate Plant Tool Kit (Figure 3A and Supporting Information, Data 2 and 3).

The Golden Gate MoClo Plant Parts Kit. A range of coding and regulatory sequences of plant, viral, and bacterial origin were domesticated for Golden Gate cloning. The resultant parts are made available as level zero modules (Figure 3B and Supporting Information, Data 4 and 5). Parts were tested by assembly into transcription units containing GFP as a reporter and evaluated by transient expression in *Nicotiana benthamiana* leaves (Supporting Information, Data 6−10). Twenty-one tested promoters (including three versions of the 35S promoter from Cauliflower Mosaic Virus) provide a range of expression strengths, with the highest levels of expression obtained with the 35S promoter, and the lowest with the maize *Spm* promoter (Supporting Information, Data 6). By comparison, the six 5′ untranslated sequences of plant and viral origins tested did not have as strong an effect on the level of GFP in *N. benthamiana* leaves (Supporting Information, Data 7). Finally, 14 tested terminators (modules 3U+Ter, 10 of which are included in the parts kit) were found to influence the quantity of GFP (Supporting Information, Data 8) even though all transcription units were under control of the 35S promoter.

In addition to regulatory sequences, 30 modules containing coding sequences are also supplied as module types 5U+NT1, NT1, SP, CDS1, CDS1 ns, and CT. These parts provide a range of subcellular localization signals, antigenic tags, and reporters. Localization signals include a synthetic chloroplast targeting peptide, a mitochondrial targeting peptide derived from the *CoxIV* gene from *Saccharomyces cerevisiae*, a signal peptide from the amylase gene from *Oryza sativa*, and a nuclear localization signal derived from Simian Virus 40. These modules were tested by constructing transcription units containing GFP as a reporter and observing the localization patterns in *N. benthamiana* leaves (Supporting Information, Data 9). Modules designed to add an N-terminal polyhistidine tag to cytosol-, apoplast- or chloroplast-targeted coding sequences are also provided. The inclusion of this tag did not affect the intracellular localization of GFP (Supporting Information, Data 9). Finally, several domesticated antigenic tags and fluorescent reporters were also assessed by transient expression in *N. benthamiana* leaves (Supporting Information, Data 10).

CONCLUSION

Sequences that confer transcriptional control, mRNA stability and translational control are valued resources in plant biotechnology. While strong constitutive and well-proven gene regulators of viral and bacterial origins are useful for many applications, a choice of strength and specificity is required. We provide here a starter kit for plant synthetic biology that contains a set of domesticated regulatory and coding sequences. We present test data for a single species, but the contents of the kits described here can be used to build single and multigene vectors to test parts and engineer proteins in many plants systems.

dx.doi.org/10.1021/sb4001504 | *ACS Synth. Biol.* 2014, 3, 839−843

Figure 3. (A) Golden Gate MoClo Plant Tool Kit contains all vector backbones and sequences required for domestication of new sequences and assembly of single and multigene binary constructs. It includes a universal level −1 cloning vector to facilitate the cloning of large level zero modules; level zero acceptors for each type of module; level one binary vectors for assembly of complete transcriptional units; level two, M, and P binary vectors and end-linkers for the construction of multigene constructs. Gray = kanamycin resistance; green = spectinomycin resistance; blue = carbenicillin resistance. Vector sequences and a description of vectors are provided in Supporting Information, Data 2 and 3. (B) The Golden Gate MoClo Plant Parts Kit consists of 96 parts provided as spectinomycin resistant level zero modules. The kit contains promoters, 5′ untranslated leaders, N-terminal tags, signal peptides, C-terminal tags, and coding sequences of synthetic, plant-viral, bacterial, and plant origin. Backgrounds are colored by module type. Vector sequences and a description of each module are provided in Supporting Information, Data 4 and 5.

To make domesticated modules as widely useful as possible, it is desirable that users conform to a single assembly standard. A number of different Golden Gate cloning vectors for plants have been published.[10,12−14] To try to avoid the creation of multiple standards, the sequences of the fusion sites chosen for the novel modules described here were jointly agreed between the laboratories contributing to this work and the developers of GoldenBraid.[13] However, despite these efforts, GoldenBraid modules still differ from the standard described here in both the identity of the type IIS enzymes used at the various cloning levels and the antibiotic markers used in the vector backbones. Therefore, use of the modules from one system in the other system would still require a conversion step.

The assembly standard described here for plants could also be applied to any other class of organisms, including mammals, fish, insects, fungi, and bacteria. The level −1 and zero cloning vectors provided in the Plant Tool Kit can be used to make basic parts for any of these organisms. Moreover, many of the

dx.doi.org/10.1021/sb4001504 | ACS Synth. Biol. 2014, 3, 839−843

tags and reporters provided in the Parts Kit are commonly used in nonplant species. These parts will be directly applicable to multiple systems, provided that the same assembly standard is applied and that transfection vectors in which transcriptional units can be assembled (levels one, two, M, and P) are constructed for each class of organism.

METHODS

A. tumefaciens strains containing level one binary vectors were grown at 28 °C overnight in LB medium supplemented with rifampicin and carbenicillin (all 50 μg/mL). The cultures were diluted to an OD_{600} of 0.2 in infiltration solution containing 10 mM MES pH 5.5 and 10 mM $MgSO_4$ and were infiltrated in leaves of greenhouse-grown *N. benthamiana* plants using a syringe without a needle. Leaves were imaged 4 days after infiltration or GFP extracted for quantification 5 days after infiltration.

ASSOCIATED CONTENT

Supporting Information

Contents and sequences of the Golden Gate MoClo Plant Tool Kit, contents, and sequences of the Golden Gate MoClo Plant Parts Kit, a description of how to construct new level zero modules of all types, and performance data of modules provided in the Golden Gate Plant Parts Kit. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: nicola.patron@tsl.ac.uk.

*E-mail: smarillo@ipb-halle.de.

Present Address

[‖]Nomad Bioscience GmbH, Biozentrum Halle, Weinbergweg 22, D-06120 Halle (Saale), Germany,

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to Hagen Stellmach and Bettina Hause for helping with the confocal microscopy pictures.

REFERENCES

(1) Kahl, L. J., and Endy, D. (2013) A survey of enabling technologies in synthetic biology. *J. Biol. Eng.* 7, 13.

(2) Anderson, J. C., Dueber, J. E., Leguia, M., Wu, G. C., Goler, J. A., Arkin, A. P., and Keasling, J. D. (2010) BglBricks: A flexible standard for biological part assembly. *J. Biol. Eng.* 4, 1.

(3) Shetty, R. P., Endy, D., and Knight, T. F. (2008) Engineering BioBrick vectors from BioBrick parts. *J. Biol. Eng.* 2, 5.

(4) Shetty, R., Lizarazo, M., Rettberg, R., and Knight, T. F. (2011) Assembly of BioBrick standard biological parts using three antibiotic assembly. *Methods Enzymol.* 498, 311−326.

(5) Sleight, S. C., Bartley, B. A., Lieviant, J. A., and Sauro, H. M. (2010) In-Fusion BioBrick assembly and re-engineering. *Nucleic Acids Res.* 38, 2624−36.

(6) Xu, P., Vansiri, A., Bhan, N., and Koffas, M. A. G. (2012) ePathBrick: a synthetic biology platform for engineering metabolic pathways in E. coli. *ACS Synth. Biol.* 1, 256−66.

(7) Engler, C., Kandzia, R., and Marillonnet, S. (2008) A one pot, one step, precision cloning method with high throughput capability. *PLoS One* 3, e3647.

(8) Ellis, T., Adie, T., and Baldwin, G. S. (2011) DNA assembly for synthetic biology: from parts to pathways and beyond. *Integr. Biol. (Camb)* 3, 109−18.

(9) Weber, E., Engler, C., Gruetzner, R., Werner, S., and Marillonnet, S. (2011) A modular cloning system for standardized assembly of multigene constructs. *PLoS One* 6, e16765.

(10) Sarrion-Perdigones, A., Falconi, E. E., Zandalinas, S. I., Juárez, P., Fernández-del-Carmen, A., Granell, A., and Orzaez, D. (2011) GoldenBraid: an iterative cloning system for standardized assembly of reusable genetic modules. *PLoS One* 6, e21622.

(11) Werner, S., Engler, C., Weber, E., Gruetzner, R., and Marillonnet, S. (2012) Fast track assembly of multigene constructs using Golden Gate cloning and the MoClo system. *Bioeng. Bugs* 3, 38−43.

(12) Emami, S., Yee, M.-C., and Dinneny, J. R. (2013) A robust family of Golden Gate Agrobacterium vectors for plant synthetic biology. *Front. Plant Sci.* 4, 339.

(13) Sarrion-Perdigones, A., Vazquez-Vilar, M., Palací, J., Castelijns, B., Forment, J., Ziarsolo, P., Blanca, J., Granell, A., and Orzaez, D. (2013) GoldenBraid2.0: A comprehensive DNA assembly framework for Plant Synthetic Biology. *Plant Physiol.* 162, 1818−31.

(14) Lampropoulos, A., Sutikovic, Z., Wenzl, C., Maegele, I., Lohmann, J. U., and Forner, J. (2013) GreenGate - A Novel, Versatile, and Efficient Cloning System for Plant Transgenesis. *PLoS One* 8, e83043.

NOTE ADDED AFTER ASAP PUBLICATION

This manuscript describes a Golden Gate resource for plant scientists and defines an assembly standard for a parallel DNA assembly method. The toolkit contains 96 Golden Gate Compatible parts, the majority of which are sequences that are already in use. While the sequences included in this paper are generally not identical to the previously published sequences (the changes being critical and necessary for use in the Golden Gate Modular Cloning Toolbox and/or for use in plants), this updated version of the paper now contains (in the Supporting Information file 4) references to the original publications that initially demonstrated the development or function of these parts.