TITLE: Multigene engineering by GoldenBraid cloning: from plants to filamentous fungi and beyond

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ABSTRACT

Many synthetic biologists have adopted methods based on Type IIS restriction enzymes and Golden Gate technology in their cloning procedures, as these enable the combinatorial assembly of modular elements in a very efficient way following standard rules. GoldenBraid (GB) is a Golden Gate-based modular cloning system that, in addition, facilitates the engineering of large multigene constructs and the exchange of DNA parts as result of its iterative cloning scheme. GB was initially developed specifically for plant synthetic biology, and it has been subsequently extended and adapted to other organisms such as Saccharomyces cerevisiae, filamentous fungi or human cells by incorporating a number of host-specific features to its basic scheme.

Here we describe the general GB cloning procedure, and provide detailed protocols of its adaptation to filamentous fungi, a GB variant known as FungalBraid. The assembly of a cassette for gene-disruption by homologous recombination, a fungal-specific extension of GB utility, is also shown. Development of FungalBraid was relatively straightforward as both plants and fungi can be engineered using the same binary plasmids via Agrobacterium-mediated transformation. We also describe the use of a set of web-based tools available at the GB website (gbcloning.upv.es), which assists users in all cloning procedures. The availability of plant and fungal versions of GB will facilitate genetic engineering in these industrially relevant organisms.

KEYWORDS

GoldenBraid, synthetic biology, modular cloning, Agrobacterium-mediated transformation, FungalBraid

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Synthetic biology and metabolic and genome engineering projects are highly dependent on having simple and fast DNA assembly techniques. Over the past two decades, several modular DNA assembly technologies, such as Gibson, SLIC or type IIS-based methods, appeared to overcome the limitations of classical cloning with TypeII restriction enzymes. Among them, Type IIS-based restriction methods were the ones that succeed. Type IIS restriction enzymes, unlike Type II enzymes, recognize nonpalindromic sequences and cut outside of their recognition site enabling the definition of these sites by the user and consequently the assembly of multiple parts in a defined order in a “one-pot” restriction-ligation reaction using only one restriction enzyme (Engler, Gruetzner, Kandzia, & Marillonnet, 2009).

GoldenBraid is a DNA assembly method that relies on the use of Type IIS restriction enzymes and allows hierarchical assemblies from individual DNA parts to complex multigenic constructs. GB assemblies involve three consecutive cloning steps, namely, domestication, multipartite transcriptional unit assemblies and binary multigene assemblies. The domestication involves the adaptation of a DNA sequence to create a standard level 0 part by (1) the removal of internal restriction sites for the two type IIS restriction enzymes used in GB assemblies (BsaI and BsmBI), (2) the addition of standard 4-nt overhangs that will define the position of these parts in the TU and (3) the cloning of the GB-adapted PCR products in the Universal Part Domestication Plasmid (pUPD2) with a BsmBI restriction-ligation reaction (Figure 1A). In GB, any new DNA part cloned in the pUPD2 is referred to as Level 0 part or phytobrick. Phytobricks belonging to the same category, that is flanked by the same standard 4-nt overhangs upon BsaI restriction, and with the same function in the TU (i.e. promoters, CDS or terminators) are fully exchangeable among the GB and

the Golden Gate plant communities. This is a result of the definition of a common grammar defining the most common categories of parts and the most common TU structures (Figure 1B) (Patron et al., 2015).

After domestication, Level 0 parts with compatible overhangs are assembled in a one-pot BsaI multipartite reaction to create a transcriptional unit or Level 1 TU in a GoldenBraid destination vector (pDGB) (Figure 1C). Next, using a set of only 4 pDBs designed to introduce a double loop (braid) into the cloning strategy, GB enables virtually limitless assembly of these transcriptional units into multigene constructs (Level >1) following an iterative cloning approach. Each pDGB contains the selection LacZ gene flanked by two restriction sites corresponding to two different type IIS enzymes, BsaI and BsmBI. Disposition of BsaI and BsmBI sites in each plasmid defines pDGB α-level and pDGB Ω-level plasmids, which are used for the BsaI- and BsmBI-GoldenBraid reactions, respectively. These plasmids also differ in their resistance marker, kanamycin for level α and spectinomycin for level Ω, allowing counterselection (Figure 1D). To ensure an endless cloning schema, a minimum set of four pDGBs is required (pDGBα1, pDGBα2, pDGBΩ1, and pDGBΩ2) (Sarrion-Perdigones et al., 2013). Additionally, this set can be expanded to eight plasmids to enable assemblies in different orientations (pDGBα1R, pDGBα2R, pDGBΩ1R, and pDGBΩ2R). The "R" on the names of these plasmids stands for reverse since these vectors are equivalent to pDGBα1, pDGBα2, pDGBΩ1, and pDGBΩ2 respectively, but designed so any TU/module assembled with them will be in reverse orientation. Two complete sets of binary vectors are available for Agrobacterium-mediated plants and fungi transformation, one based on the pGreenII backbone (the pDGB1 set) and another set based on the pCAMBIA backbone (the pDGB3 set).

GB comprises a library of DNA parts (phytobricks) used to compose multigene constructs for metabolic engineering, genome engineering and other synthetic biology applications. Among other elements, this collection includes (i) a collection of plant promoters and terminators with documented transcriptional activities (Sarrion-Perdigones et al., 2013), (ii) a selection of inducible gene expression systems, such as dexamethasone, estradiol or ethanol (Vazquez-Vilar et al., 2017), (iii) a number of Cas9 and Cas12a phytobricks for genome editing (Bernabé-Orts et al., 2019; Vazquez-Vilar et al., 2016), and (iv) a collection of dCas9-based programmable transcriptional activators for gene regulation (Selma et al., 2019).

Although GB was initially developed for nuclear transformation in plants (Sarrion-Perdigones et al., 2011), it has been adapted since then for its use in plastids (Vafaee, Staniek, Mancheno-Solano, & Warzecha, 2014), yeast (Pérez-González et al., 2017) and filamentous fungi (Hernanz-Koers et al., 2018), and human cells (Sarrion-Perdigones et al., 2019). The creation of new parts (i.e. promoters, new codon optimized CDSs...) is usually enough to make GB fully functional for transfection in any other organisms besides plants, fungi and human cells. Additionally, the modularity of GB allows for the construction of cassettes for targeted genome integration or for gene disruption in a time-efficient and easy way. Three alternative cloning strategies have been developed based on GB, each of them designed to better suit the specificities of the organism it was developed for. Two of these strategies involved the use of standard GB vectors and syntax, either in multipartite reactions (Hernanz-Koers et al., 2018) or with subsequent binary assemblies (Vafaee et al., 2014). The third strategy was designed for *Saccharomyces cerevisiae*, which has well-characterized loci conferring high level expression of transgenes (Flagfeldt, Siewers, Huang, & Nielsen, 2009). This latter strategy involved backbone adaptation in order to create two sets of GB destination plasmids, each of them containing a pair of widely used homology arms adjacent to the GB-cassette (Pérez-González et al., 2017). This variety of strategies and some of their

published applications are summarized in **Table 1,** and show the suitability of GB to accommodate the specificities of organisms other than plants.

The use of a common GB syntax enables the trans-kingdom exchange of DNA parts. Only recently, similar methods have emerged that, following the same trans-kingdom approach, allow for the exchange and assembly of DNA parts derived from distant organisms (Chiasson et al., 2019). Here, we describe protocols for the assembly and transformation of multigene constructs in filamentous fungi, which show how GB workflow can be extended beyond plants. Some of said constructs comprise elements previously developed for plants, illustrating the exchangeability of parts between distant organisms.

Basic Protocol 1 outlines the software-assisted assembly of a 2X transcriptional unit (TU) plasmid for constitutive yellow fluorescent protein (YFP) expression in filamentous fungi. **Basic Protocol 2** describes the transformation of the newly generated constructs plasmids in *Penicillium digitatum* (PDMH021 strain); *Penicillium expansum* (PEMG041 and PEMG154 strains) and *Aspergillus niger* (ANEM001 strain) for assessing the functionality of the chosen DNA regulatory elements in these organisms. The protocol requires lab equipment available in any molecular biology laboratory and a computer with internet access for *in silico* design of the intended assembly.

Basic Protocol 3 provides a detailed software-assisted protocol for the modular assembly of a gene disruption-cassette targeting the *msb2* gene. **Basic Protocol 2** explains how the transformation of this cassette can be performed and **Basic Protocol 4** specifies the screening process of gene deletion mutants.

STRATEGIC PLANNING

The straightforwardness of the GB cloning workflow described in **Basic Protocol 1** added to a careful design of the intended construct, including appropriate selection of the destination vector depending on the number of Tus to assemble, is crucial to minimize cloning steps, thus saving time and effort. **Basic Protocol 1** describes the assembly of two TUs. The assembly and fungal expression of a 2X TU expression cassette takes 21 days in total (see **Figure 2**). The assembly of 3-4 TUs involves an extra step in the GB loop (from Ω-level to α-level) and therefore, three extra days for the cloning process (**Figure 2**). Each time the number of TUs is doubled, e.g. from 3-4 to 6-8 TUs, an extra step in the GB loop, with its associated three extra cloning days, is needed. However, careful planning, keeping in mind the intended final assembly, can speed up the cloning process as multiple assemblies in the correct destination vectors can be constructed in parallel. Although knowing the final assembly will speed up the process, the major advantage of the GB cloning system is its versatility. Since each part can be re-used, assemblies with additional or different combinations of parts and/or TUs can be efficiently added.

The GB cloning platform includes gbcloning, a specific web-based software for multigene constructs design hosted at gbcloning.upv.es website (Vazquez-Vilar et al., 2017). Gbcloning assists users starting from the DNA sequence adaptation to the GB standards through to the assembly of transcriptional units and multigene constructs as described below in **Basic Protocol 1**. The *in silico* assembly of the final construct prior to its assembly in the laboratory minimizes the possibility of errors during primer design or destination vector selection, speeding up the whole assembly process. Gbcloning also includes a database of public genetic elements generated with GB that can be incorporated into any user's designs (available at Addgene repository or under demand), thus favoring the reusability and minimizing the amount of work required to generate the desired assembly. In order to store and reuse any newly created GBelement, a 'user login' is required. A new account can be easily created at https://gbcloning.upv.es/accounts/register/.

BASIC PROTOCOL 1

Basic Protocol 1 Title: Software-assisted modular DNA assembly of a two gene expression-cassette with GB

Basic Protocol 1 Introduction

In this protocol, we will describe a construct to obtain YFP-labelled fungal strains. The YFP from the GB repository (GB0053 element) was assembled into pDGBα1R under the control of a fungal-specific promoter (FB007 element, P*gpdA* from *Aspergillus nidulans*) and terminator (FB008 element, T*trpC* from *A. nidulans*) to constitutively express the fluorescent protein. The resulting TU (FB026) that expresses the fluorescent protein was combined with a positive Hyg^R selection marker (FB003 element) to obtain the FB027 element (**Figure 3**). Similarly, the YFP-TU has been assembled with the positive selection marker $G418^R$ (FB009 element) into the pDGBΩ1 binary vector (FB039 element). Both binary vectors (FB027 and FB039) have been used to transform *P. digitatum* (PDMH021 strain), *P. expansum* (PEMG041 and PEMG154 strains) and *A. niger* (ANEM001 strain) to generate different YFP-tagged strains (**Figure 4**). The constitutive expression of any other gene of interest could be achieved with a similar approach, changing the YFP for the specific gene.

Although the protocol below describes the assembly of a multigene construct for fungal transformation, the general procedure for GB assemblies is the same regardless of the origin and the final application of the assembled DNA elements.

Basic Protocol 1 Materials list

- Equipment
    1. Thermocycler
    2. Agarose gel electrophoresis equipment
    3. Incubator (temperature-controlled)
    4. Shaking incubator (temperature-controlled)
    5. Computer with access to the internet (www.gbcloning.upv.es).

- DNA elements
    1. Universal parts domesticator plasmid or GB entry plasmid for Level 0 parts (pUPD2, https://www.addgene.org/68161/).
    2. GB-adapted primers and template for PCR amplification of the genetic sequence to be adapted to the GB standard (see **Figure 3A** and **Table 2**).
    3. Level 0 parts: GB0053 (pYFP, https://www.addgene.org/68197/), FB008 (T*trpC*, https://www.addgene.org/119706).
    4. Level 1 parts: FB003 (P*trpC*:*hph*:T*tub*, https://www.addgene.org/119677/).
    5. Destination plasmids: pDGB3alpha1R (https://www.addgene.org/68230/) and pDGB3omega1 (https://www.addgene.org/68238/).

- Enzymes and solutions
    1. AccuPrime® *Taq* DNA polymerase High Fidelity (Invitrogen #12346086).
    2. 10x AccuPrime® PCR-buffer (Invitrogen #12346086).
    3. BsaI from New England Biolabs (10000 U/mL #R0535S).
    4. BsmBI from New England Biolabs (10000 U/mL #R0580S).

5. T4 DNA Ligase from Promega (1-3 Weiss units/µl #M1804).
6. Ligase 10x buffer from Promega (300 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT and 10mM ATP #M1804).
7. NotI from Thermo Scientific (10 U/µl #ER0595).
8. Buffer O 10X from Thermo Scientific (#BO5).
9. BamHI from Thermo Scientific (#ER0051).
10. Buffer BamHI 10X from Thermo Scientific (#B57).
11. HindIII from Thermo Scientific (10 U/µl #ER0501).
12. Buffer R from Thermo Scientific (#BR5).
13. BSA (Bovine serum albumin): Prepare as stock of 1 mg/mL in H_2O.
14. Agarose. Agarose gels are prepared with 1% agarose and 0.001% ethidium bromide in TAE 1X.
15. TAE 1X (40 mM Tris-acetate and 1 mM EDTA).

● Commercial kits

1. Mix & Go! E. coli Transformation Kit and Buffer Set (Zymo Research).
2. E.Z.N.A.® Plasmid Mini Kit I, (Q-spin) (Omega Bio-Tek).
3. QIAprep Spin Miniprep Kit (Qiagen).
4. NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel).

● Culture media

1. Luria-Bertani (LB) broth: 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl.
2. Luria-Bertani (LB) agar: LB broth with 15 g/L of bacteriological agar.

● Antibiotics and other chemical agents (see recipe in Reagents and Solutions section)

1. Spectinomycin stock solution (50 mg/mL).
2. Kanamycin stock solution (50 mg/mL).
3. Chloramphenicol stock solution (34 mg/mL).
4. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) stock solution (20 mg/mL).
5. IPTG (Isopropyl-β-D-thiogalacto- pyranoside) stock solution (0.4 M).
6. Ethidium bromide stock solution.

Basic Protocol 1 steps and annotations

Level 0 DNA part assembly

The first step in the GB cloning schema is the adaptation of the new genetic sequence to the GB standard in a process known as domestication or Level 0 part assembly in the universal parts domestication plasmid (pUPD2). This process involves the addition of part specific overhangs (see **Table 2**), the removal of internal BsaI and BsmBI sites and the cloning of the generated PCR products in the pUPD2 vector.

Step 1. The domestication of any genetic sequence to the FungalBraid (FB) grammar is software-assisted at https://gbcloning.upv.es/fungal/do/domestication/. Select 'Fungal_PROM+5UTR (A1-A2-A3-B1-B2)' as the category to domesticate and upload a FASTA file with the P*gpdA* promoter sequence. Click on submit and download the protocol containing the primers listed in **Table 3**

(**Figure 3 A**). Upload the newly generated part to the GB database by clicking on 'Add part' and filling in the required fields in order to use it in Step 8 for the TU assembly.

Step 2. PCR amplify the plasmid pLY870 as template with primers obtained in Step1 (P*gpdA* Fwd and P*gpdA* Rev) following AccuPrime specifications.

Step 3. Perform agarose gel electrophoresis to verify the PCR product and PCR purification following the manufacturer's indications (NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel)).

PCR purification is not essential when the cloning involves the assembly of a single PCR product in the pUPD2 plasmid. However, this step is recommended to get rid of the primers. When primer dimers are observed in the agarose gel, gel purification is advisable. Primer dimers are cloned with higher efficiency than larger PCR products.

Step 4. Set up the restriction-ligation reaction in 15 µl with 60 fmoles of the purified PCR product, 20 fmoles of pUPD2, 1.5 µl of 10x Ligase buffer, 1.5 µl of BSA (stock 1mg/ml), 0.8 µl of BsmBI and 0.8 µl of T4 Ligase. Incubate the reaction using the following program: 10' 37 ºC + 25x (3' 37 ºC + 4' 16 ºC) + 10' 50 ºC + 10' 80 ºC.

Femtomoles are calculated based on formula: moles of dsDNA (mol) = mass of dsDNA (g) / (length of dsDNA (bp) * 617.96 g/mol) +36.04 g/mol (https://nebiocalculator.neb.com/#!/formulas).

Steps at 37 ºC during the restriction-ligation reaction favor the restriction of the initial parts while steps at 16 ºC favor ligation of previously cut parts. The 50 ºC step favors restriction of undigested initial parts reducing the background of blue colonies on the plates. The final step at 80 ºC is to inactivate the enzymes.

Step 5. *Escherichia coli* JM109 transformation with the ligation product from Step 4. Spread on plates with 34 mg/L chloramphenicol, 0.4 mM IPTG and 40 mg/L X-gal. Incubate at 37 ºC overnight.

Step 6. Pick four white colonies in 2 ml of LB broth with 34 mg/L chloramphenicol. Grow overnight at 37 ºC in a shaking incubator.

Screening of two colonies is usually enough to find at least one with the intended assembly. However, for Level 0 parts we recommend to screen four colonies since some of them might have point mutations in the amplified sequence (see Step 7).

Step 7. Extract plasmids and perform *Not*I restriction enzyme digestion for correct assembly verification with 400 ng of DNA, 1.5 µl of 10x Buffer O and 0.5 µl of NotI in a final volume of 15 µl. Incubate the reaction at 37 ºC for 1 hour. Analyze samples of restriction digest, along with a DNA ladder, by agarose gel electrophoresis to verify that the DNA banding pattern is the expected one (expected bands: 2046-791 bp). Sanger sequencing of the positive plasmids is essential at this step to check for any unintended mutations occurred during the PCR amplification step. The Genbank file of the generated plasmid pUPD2_ P*gpdA* can be downloaded from the part stored in gbcloning at Step 1. This file can be opened for visualization, restriction analysis planning and sequence alignment with the desired sequence manager software.

Any sequence manager software can be used for planning restriction analysis and aligning the Sanger sequencing results with the theoretical sequence. We recommend using Benchling (https://benchling.com/), *a cloud-based platform including software tools for DNA sequence design.*

Level 1 TU multipartite assembly

The next step on the GB schema is the assembly of TU or Level 1 elements in a multipartite reaction with Level 0 elements and any pDGB3α as the destination vector.

Step 8. Go to https://gbcloning.upv.es/fungal/do/multipartite/, click on the 'Basic TU' option and select the pUPD2_ P*gpdA* GBpart uploaded to the database in Step 1 as the 'Promoter', as 'CDS' pYFP (GB0053) and as 'Ter' pT*trpC* (FB008). Choose the pDGB3α1R as the 'Vector'. Upload the generated TU to the database by clicking on 'Add part' and filling in the required fields (**Figure 3 B**).

Step 9. Set up Level 1 restriction-ligation in 15 µl with 20 fmoles of each Level 0 part, 20 fmoles of pDG3α1R, 1.5 µl of 10x ligase buffer, 1.5 µl of BSA (stock at 1 mg/mL), 0.8 µl of BsaI and 0.8 µl of T4 DNA Ligase. Incubate the reaction using the following program: 10' 37 ºC + 25x (3' 37 ºC + 4' 16 ºC) + 10´ 50 ºC + 10' 80 ºC.

Step 10. Transform *E. coli* JM109 with the ligation product from Step 9. Spread on plates with 50 mg/L kanamycin, 0.4 mM IPTG and 40 mg/L X-gal. Incubate at 37 ºC overnight.

Step 11. Pick two white colonies in 2 ml of LB broth with 50 mg/L kanamycin. Grow overnight at 37 ºC in a shaking incubator.

Step 12. Extract plasmids and set up a restriction analysis reaction with 400 ng of plasmid, 1.5 µl of 10X Buffer BamHI and 5 U of BamHI in 15 µl of final volume for correct assembly verification. The Genbank file of the generated plasmid can be downloaded from the part stored at gbcloning in Step 8 for planning the restriction analysis. Analyze samples of restriction digest, along with a DNA ladder, by agarose gel electrophoresis to verify that the DNA banding pattern is the expected one (expected bands: 6346-1744 bp).

Level >1 genetic module binary assembly

Step 13. Go to https://gbcloning.upv.es/do/bipartite/ and select the TU uploaded to the GB database in Step 8 as 'Part 1'. Select the FB003 element (Hyg^R TU) as 'Part 2' and the pDG3Ω1 vector as 'Vector'. Store the generated module in the database by clicking on 'Add part' and filling in the required fields (**Figure 3 C**).

Step 14. Set up Ω-Level restriction-ligation in 15 µl with 20 fmoles of each α-Level plasmid containing a TU, 20 fmoles of pDG3Ω1, 1.5 µl of 10x ligase buffer, 1.5 µl of BSA (stock at 1 mg/mL), 0.8 µl of BsmBI and 0.8 µl of T4 DNA Ligase. Incubate the reaction with the following program: 10' 37 ºC + 25x (3' 37 ºC + 4' 16 ºC) + 10´ 50 ºC + 10' 80 ºC.

Step 15. Transform *E. coli* JM109 with the ligation product from Step 14. Spread on plates with 50 mg/L spectinomycin, 0.4 mM IPTG and 40 mg/L X-gal. Incubate at 37 ºC overnight.

Step 16. Pick two white colonies in 2 ml of LB broth with 50 mg/L spectinomycin. Grow overnight at 37 ºC in a shaking incubator.

Step 17. Extract plasmids and set up a restriction analysis reaction with 400 ng of plasmid, 1.5 µl of 10X Buffer BamHI and 5 U of BamHI in 15 µl of final volume for correct assembly verification. The Genbank file of the generated plasmid can be downloaded from the part stored at gbcloning in Step 13 for planning the restriction analysis. Analyze samples of restriction digest, along with a DNA ladder, by agarose gel electrophoresis to verify that the DNA banding pattern is the expected one (expected bands: 6675-3424 bp).

BASIC PROTOCOL 2

Agrobacterium tumefaciens-mediated transformation of filamentous fungi

Introduction

Agrobacterium tumefaciens is a phytopathogenic bacteria capable of transferring DNA to different cells. This ability has been applied in several fields for the transformation of different organisms, including filamentous fungi. We present a detailed protocol modified from Khang et al. (Khang, Park, Rho, Lee, & Kang, 2006) and Michielse et al. (Michielse, Hooykaas, van den Hondel, & Ram, 2008) to transform different filamentous fungi, such as *P. digitatum*, *P. expansum* or *A. niger* using *A. tumefaciens*-mediated transformation (ATMT).

Basic protocol 2 material list

- Equipment
    1. Sterile Petri plates 90 mm and 50 mm (Thermo Fisher)
    2. Shaker incubator for *Agrobacterium* and fungal plates/culture (temperature-controlled)
    3. electroporation cuvettes
    4. Gene Pulser X-Cell System BioRad (Ref: 1652660)
    5. Spectrophotometer and plastic cuvettes
    6. Hemocytometer
    7. Miracloth
    8. Laminar flow cabinet

- Culture media
  All solid culture media use 1.5% agar (Pronadisa; Madrid, Spain) to solidify medium and are autoclaved (120°C for 20 min) before use
    1. SOC medium: 20 g/L of tryptone, 5 g/L of yeast extract, 0.5 g/L NaCl, 0.2 g/L KCl. Adjust to pH 7. Autoclave and add 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose.
    2. LB broth: 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl.
    3. Potato dextrose agar: 39 g of potato dextrose agar/L (Difco; Sparks, MD)
    4. LB broth: 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl.
    5. Minimal medium (MM): See Table 4
    6. Induction medium (IM): See Table 4
    7. Co-cultivation medium (CM): See Table 4

- Plasmids, fungal and bacterial strains
    1. Binary vector containing the GB/FB element of interest
    2. *P. digitatum* CECT 20796 (Marcet-Houben et al., 2012)
    3. *P. expansum* CMP1/PEX1 (Ballester et al., 2015)
    4. *A. niger* CECT 2775
    5. *A. tumefaciens* AGL1 electrocompetent cells
    6. *E. coli* JM109

- Antibiotics and other chemical agents (see recipe in Reagents and Solutions section)
    1. Spectinomycin stock solution (50 mg/mL).
    2. Kanamycin stock solution (50 mg/mL).
    3. Rifampicin stock solution (10 mg/mL).
    4. Acetosyringone (AS) (3,5-dimethoxy-4-hydroxyacetophenone) stock solution (200 mM).

5. Hygromycin B (hygB): Ready to use solution of 100 mg/mL (Invivogen).
6. Geneticin (G418): Ready to use solution of 100 mg/mL (Invivogen).
7. Cefotaxime, sodium salt stock solution (200 mM).
8. Moxalactam, sodium salt stock solution (100 mg/mL).
9. F2dU (5-fluoro-2'-deoxyuridine) stock solution (10 mM).

- Stock Solutions (see recipe in Reagents and Solutions section)
  1. K- Buffer.
  2. Solution A (K_2HPO_4).
  3. Solución B (KH_2PO_4).
  4. MN- Buffer.
  5. 1% $CaCl_2$.
  6. 20% NH_4NO_3.
  7. 20% glucose.
  8. 0.01% FeSO4.
  9. 1M- MES.
  10. Mineral Elements.

Basic protocol 2 steps and annotations

***Agrobacterium tumefaciens* transformation with a binary vector**

Step 1. Thaw an aliquot of 40 µL of AGL1 electrocompetent cells on ice and add 1 µg of binary vector containing the GB/FB element of interest created in BP1.

Step 2. Mix well with a pipette and maintain on ice 5 minutes.

Step 3. Transfer the mixture into pre-chilled electroporation cuvette. It is very important to avoid the formation of bubbles in this step.

Step 4. Insert the cuvette into the device and electroporate at 2500 V 5 ms.

Step 5. Immediately add 960 µL of SOC medium and transfer to a sterile tube. Incubate at 28 ºC 250 rpm for 3 hours to grow cells.

Step 6. Spread cells on LB agar plate containing 20 µg/mL rifampicin and 50 µg/mL kanamycin (for pDGBα vectors) or 100 µg/mL spectinomycin (for pDGBΩ vectors) and incubate the plates 48 hours at 28 °C until transformant colonies appear.

***Agrobacterium tumefaciens*-mediated transformation of filamentous fungi**

<u>Preparation of fungal spores</u>

Different steps are summarized in the **Figure 5**

Step 7. Inoculate aliquot of glycerol stock of fungal spores on PDA medium, distributing the solution throughout the plate with a sterile glass rod.

Step 8. Incubate during 5-7 days in the dark at 25 ºC until spores appear. Incubation times depend on sporulation of fungal species. For each transformation assay, it is necessary to have a fresh fungal plate to obtain the first young spores that appear. Young spores are needed; the use of older spores reduce transformation frequency. The incubation temperature depends on the fungi.

Step 9. After this, harvest fungal spores with a sterile spatula and resuspend in 5 mL sterilized H_2O.

Step 10. Filter the spore suspension through Miracloth to remove large debris. Repeat the filtration one more time. In some cases, it may be appropriate to centrifuge the spores for 5 min at 2,000 g at room temperature, discard the supernatant and resuspend the spores in sterilized H_2O.

Step 11. Determine the spore concentration using a hemocytometer and dilute the spores to a final concentration of 10^6 conidia/mL.

Preparation of bacteria, culture media and reagents

Step 12. Streak *A. tumefaciens* AGL1 harbouring the corresponding binary vector on an LB plate containing 20 µg/mL rifampicin (to prevent bacterial contamination) and the respective antibiotic (50 µg/mL kanamycin for pDGB3α binary vectors or 100 µg/mL spectinomycin for pDGB3Ω binary vectors).

Step 13. Incubate for 48 h at 28 ºC.

Step 14. Prepare MM medium (**Table 4**), without antibiotic, 10 mL for each transformation.

Step 15. Prepare IM medium (**Table 4**), without AS and antibiotic, 10 mL for each transformation.

Step 16. Prepare CM small Petri plates (50 mm). Six plates per transformation to obtain several replicates of transformants.

Step 17. Prepare fungal selection plates (50 mm) with PDA supplemented with the appropriate antibiotics (hygromycin) and 200 µM cefotaxim and 100 µg/mL moxalactam (to kill the *Agrobacterium*). Six plates per transformation.

Step 18. Prepare fungal selection plates (90 mm) with PDA supplemented with the appropriate antibiotic (hygromycin) to inoculate washes of the other selection plates. Six plates per transformation.

Step 19. Prepare fungal dual selection plates (negative selection) with PDA supplemented by 25 µM F2dU. Optional plates only used to obtain disruption transformants.

Step 20. Prepare fungal selection plates (24 wells) with PDA supplemented with the appropriate antibiotic (hygromycin/G418) to obtain isolate transformants.

Transformation Protocol

Step 21. Pick a single colony of *Agrobacterium* and inoculate into 10 mL of MM medium supplemented with the corresponding antibiotic (**Table 4**) using an Erlenmeyer flask of 100 mL. Incubate culture at 28 ºC with shaking (250 rpm) for 48 h. It can be prepared on Friday afternoon to induce Monday morning.

Step 22. After incubation, measure the OD at 600 nm using a spectrophotometer (it must be between 1.0-1.5).

Step 23. For *Agrobacterium* induction, centrifuge 1 mL of culture for 10 min at 1000 g at room temperature in a 1.5 mL tube. Remove the supernatant. Resuspend the pellet in 750 µL of IM medium (**Table 4**) freshly prepared with the corresponding antibiotic. Centrifuge for 5 min at 1000 g at room temperature.

Step 24. Remove the supernatant and resuspend the pellet in 500 µL of IM medium. Add the suspension into 4 mL of IM medium using a 100 mL flask. Wash the tube with 0.5 mL of new IM medium and add together into the flask in a total volume of 5 mL.

Step 25. Incubate the cultures for 5-6 hours at 28 ºC with gentle shaking at 250 rpm until reaching the OD_{600} of 0.6 (~$1x10^8$ bacterial cells/mL). Start the OD measurements after 3 h of incubation to register the growth. The time required to reach an OD_{600}= 0.6 depends on the *Agrobacterium* strain.

Step 26. During the incubation prepare co-cultivation membranes. Using sterile tweezers, place nitrocellulose membranes onto CM medium Petri plates (50mm). Moisten the membranes to promote contact with medium and avoid bubbles. Plates with membranes should be dried before spreading the co-cultivation.

Step 27. Mix 100 µL of *Agrobacterium* induced cells with OD_{600} of 0.6 from Step 5 with 100 µL of the fungal conidiospores at a concentration of $1x10^6$ conidia/mL (ratio 1:1). The optimal ratio *Agrobacterium*:fungal conidiospores must be determined empirically.

Step 28. Spread the mixture (200 µL) containing *Agrobacterium* cells and fungal spores onto CM medium Petri plates (50 mm) with nitrocellulose membranes using a sterile glass rod spreader.

Step 29. Incubate the plates for 2 days at 25 ºC. The optimal combination of temperature and incubation time depends on the fungal species and must be determined empirically.

Transformant selection

Step 30. Transfer nitrocellulose membranes with the mixture of *Agrobacterium*-fungal spores onto fungal selection plates (50 mm) supplemented with the corresponding antibiotic and 200 µM cefotaxim and 100 µg/mL moxalactam (to kill the *Agrobacterium*) using sterile tweezers. The antibiotic used for selection depends on the selection marker (FB element) used in the final assembly: G418 for FB009 and hygromycin for FB003. It is recommended to place the nitrocellulose membrane upside down on PDA selection medium containing cefotaxim and moxalactam for 5-10 minutes, to prevent bacterial growth. Drag the membrane around on the surface of the medium to remove *Agrobacterium* cells as much as possible, and subsequently flip and place the membrane on the same medium.

Step 31. Incubate the plates for 3-4 days at 25 ºC in the dark until colonies appear.

Step 32. When the fungal colonies are visible, wash each plate by adding 1 mL sterile H_2O on the surface. Disperse the fungal spores by pipetting up and down, recover the suspension and store this wash at 4 ºC.

Step 33. Inoculate 100 µL of the previous wash onto fungal selection plates (90 mm) supplemented with the antibiotic used as selectable marker. Inoculate dilutions of the previous wash (10^{-1} or 10^{-2}) in case of high fungal growth.

Obtainment of ectopic transformants

Step 34. Transfer viable transformants obtained in Step 4 to PDA medium supplemented with the appropriate antibiotic for positive selection in 24-well microtiter plates for sporulation. Only transformant strains that contain the specific gene marker used to discriminate (*hph*), survive and the rest can not grow.

Step 35. Use spores from the transformants grown in 24-well microtiter for molecular characterization (DNA isolation and PCR), purification and subsequent monosporic culture.

Spore suspensions can be stored in 20 % glycerol (final concentration) at -80 ºC for later use.

BASIC PROTOCOL 3

Title: Software-assisted modular DNA assembly of a gene disruption-cassette using GB

Introduction

In this protocol, we will describe the assembly of a gene disruption cassette for deletion of the *msb2* gene in *P. digitatum* CECT 20796 (**Figure 6**). This gene encodes a highly glycosylated transmembrane protein that modulates signaling pathways. Msb2 signaling mucin is a regulator of environmental stress or cell wall biogenesis and is required for filamentous growth through activation of the cell wall integrity pathway (CWI) in fungi, which controls invasive growth and infection-related morphogenesis (Gurgel et al., 2019; Pérez-Nadales & di Pietro, 2011).

Although the protocol below describes the assembly of a gene disruption construct for *P. digitatum*, the general procedure for the assembly of a gene disruption cassette targeting any other gene in any other fungi would be identical.

Basic protocol 3 materials list

- DNA elements
    1. Universal parts domesticator plasmid or GB entry plasmid for Level 0 parts (pUPD2, https://www.addgene.org/68161/).
    2. GB-adapted primers and template for PCR amplification of the genetic sequence to be adapted to the GB standard (see **Table 5**).
    3. Level 0 parts: FB012 (https://www.addgene.org/119709/), FB013 (https://www.addgene.org/119710/).
    4. Destination plasmids: pDGB3α2 (https://www.addgene.org/68229/).

● Enzymes and solutions
Listed on Basic Protocol 1
● Commercial kits
Listed on Basic Protocol 1
● Equipment
Listed on Basic Protocol 1
● Culture media
Listed on Basic Protocol 1
● Antibiotics and other chemical agents
Listed on Basic Protocol 1

Basic Protocol 3 steps and annotations

Level 0 DNA parts assembly

The first step in the GB cloning schema is the adaptation of the new genetic sequence to the GB standard in a process known as domestication or Level 0 part assembly in the universal part

domestication plasmid (pUPD2). This process involves the addition of part-specific overhangs (see **Table 2**), the removal of internal BsaI and BsmBI sites and the cloning of the generated part in the pUPD2 vector.

Step 1. The domestication of any genetic sequence to the FB grammar is software-assisted at https://gbcloning.upv.es/fungal/do/domestication/. Select 'Flank_5prime (B1-B2)' as the category to domesticate and upload a FASTA file with the 5' flanking sequence of *msb2*. Click on submit and download the protocol containing the primers listed in **Table 5**. Do the same with the 3' flanking sequence of *msb2*, but choose in this case 'Flank_3prime (B6-C1)' as the category to domesticate. Upload the two newly generated parts to the GB database by clicking on 'Add part' and filling in the required fields in order to use them in Step 8 for the TU assembly.

Step 2. PCR amplify with primers designed on Step 1 (*msb2* 5'FS Fwd + *msb2* 5'FS Rev and *msb2* 3'FS Fwd + *msb2* 3'FS Rev) following AccuPrime specifications and using *P. digitatum* CECT 20796 genomic DNA as the template.

Step 3. Purify the two PCR products following the manufacturer's indications (NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel)).

Step 4. Set up the two restriction-ligation reactions in 15 µl; each of them with 60 fmoles of the purified PCR product, 20 fmoles of pUPD2, 1.5 µl of 10x Ligase buffer, 1.5 µl of BSA (stock 1 mg/ml), 0.8 µl of BsmBI and 0.8 µl of T4 Ligase. Incubate the reactions using the following program: 10' 37 ºC + 25x (3' 37 ºC + 4' 16 ºC) + 10´ 50 ºC + 10' 80 ºC.

Step 5. Transform *E. coli* JM109 with each of the ligation products from Step 4. Spread on plates with 34 mg/L chloramphenicol, 0.4 mM IPTG and 40 mg/L X-gal. Incubate at 37 ºC overnight.

Step 6. Pick four white colonies for each assembly in 2 ml of LB broth with 34 mg/L chloramphenicol. Grow overnight at 37 ºC in a shaking incubator.

Step 7. Extract the plasmids and set up a restriction analysis reaction with 400 ng of plasmid, 1.5 µl of 10X Buffer O and 5 U of NotI in 15 µl of final volume for correct assembly verification. Analyze samples of restriction digest, along with a DNA ladder, by agarose gel electrophoresis to verify that the DNA banding pattern is the expected one (expected bands: 2046-1064 bp both for pUPD2_ msb2-5'FS and pUPD2_ msb2-3'FS). Sanger sequencing of the positive plasmids is essential at this step to check for any unintended mutations which may have occurred during the PCR amplification step that could prevent the homologous recombination process. The Genbank file of the generated pUPD2_ msb2-5'FS and pUPD2_ msb2-3'FS plasmids can be downloaded from the part stored on gbcloning in Step 1. This file can be opened for visualization, restriction analysis planning and sequence alignment with the desired sequence manager software.

Level 1 gene disruption TU multipartite assembly

Step 8. Go to https://beta.gbcloning.org/fungal/do/disruption/ and select the pUPD2_ msb2-5'FS and pUPD2_ msb2-3'FS uploaded to the database in Step 1 as the '5' flank' and '3' flank' respectively. Choose FB013 as the 'negative selection marker', FB012 as the 'positive selection marker' and the pDGB3α2 as the 'Vector'. Upload the generated TU to the database by clicking on 'Add part' and filling in the required fields.

Step 9. Set up Level 1 restriction-ligation in 15 µl with 20 fmoles of each Level 0 part, namely FB012, FB013 and the two genetic elements pUPD2_*msb2*-5'FS (FB018) and pUPD2_*msb2*-3'FS (FB019) obtained on Step 7 of this protocol, 20 fmoles of pDG3α2, 1.5 µl of 10x ligase buffer, 1.5 µl of BSA (stock at 1 mg/mL), 0.8 µl of BsaI and 0.8 µl of T4 DNA Ligase. Incubate the reaction using the following program: 10' 37 ºC + 25x (3' 37 ºC + 4' 16 ºC) + 10' 50 ºC + 10' 80 ºC.

Step 10. Transform *E. coli* JM109 with the ligation product from Step 9. Spread on plates with 50 mg/L kanamycin, 0.4 mM IPTG and 40 mg/L X-gal. Incubate at 37 ºC overnight.

Step 11. Pick two white colonies in 2 ml of LB broth with 50 mg/L kanamycin. Grow overnight at 37 ºC in a shaking incubator.

Step 12. Extract plasmids and set up a restriction analysis reaction with 400 ng of plasmid, 1.5 µl of 10X Buffer R and 5 U of HindIII in 15 µl of final volume for verification of correct assembly. Analyze samples of restriction digest, along with a DNA ladder, by agarose gel electrophoresis to verify that the DNA banding pattern is the expected one (expected bands: 6345-5494 bp).

BASIC PROTOCOL 4
Title: Obtainment of disruption transformants

Introduction

In this protocol we will describe how to obtain homologous transformants using the negative selection marker HSV*tk*, which allows for the discrimination between ectopic transformants in which the transformed DNA was randomly inserted in a region different from the targeted locus, and positive homologous transformants. The herpes simplex virus thymidine kinase (HSV*tk*) gene favours the conversion of the F2dU substrate into a toxic compound in different fungi demonstrating its use as a negative selection marker (Khang, Park, Lee, & Kang, 2005). Negative selection means that strains are selected for the loss of this HSV*tk* gene.

Basic Protocol 4 materials list
The materials and reagents needed for this protocol are listed in Basic Protocol 2.

Basic Protocol 4 steps and annotations

Step 1. Follow the Basic Protocol 2 from Step 1 to Step 33

Step 2. When the transformants from Step 33 appear, wash again the surface of each plate with 1 mL sterile H_2O. Recover and store this wash at 4 ºC.

Step 3. Inoculate 100 µL of this wash onto new fungal selection plates (90 mm) supplemented with F2dU (usually at a concentration of 25 µM). The negative selection marker HSV*tk* confers sensitivity to F2dU. Homologous recombinants lose the HSV*tk* gene and they are able to grow in the presence of F2dU because they are insensitive. Ectopic transformants expressing HSV*tk* gene fail to grow in presence of F2dU due to the presence of the negative selection gene.

Step 4. Incubate the plates for 3-5 days at 25 ºC

Step 5. Transfer viable transformants to PDA medium supplemented with appropriate antibiotics in 24-well microtiter plates for sporulation.

Step 6. Use spores from the transformants grow in the 24-well microtiter plate for molecular characterization (DNA isolation and PCR), purification and subsequent monosporic culture. **Figure 6C** and **Table 6** show the primers used for the molecular characterization of *msb2* deletion strains.

REAGENTS AND SOLUTIONS

1. Acetosyringone (AS) Prepare (3,5-dimethoxy-4 hydroxyacetophenone(acetosyringone; AS; Sigma-Aldrich, #D134406) as a stock of 200 mM in dimethyl sulfoxide (DMSO). Protect from light. Aliquot and store up to 1 year at −20°C.
2. $CaCl_2$, 1%: Dissolve 1 g of $CaCl_2.2H_2O$ in 100 mL of H_2O. Autoclave at 120°C for 20 min. Store up to 6 months at 4°C..
3. Cefotaxime, sodium salt, stock solution, 200 mM: Prepare cefotaxime, sodium salt (CalBiochem, #219380) as a stock of 200 mM in H_2O. Sterilize by filtration (0.22 µm). Aliquot and store up to 1 year at −20°C.
4. Chloramphenicol stock solution, 34 mg/mL: Prepare as a stock of 34 mg/mL chloramphenicol (Sigma-Aldrich, #C1919) in ethanol. Sterilize by filtration (0.22 µm). Aliquot and store up to 1 year at −20°C.
5. F2dU (5-fluoro-2'-deoxyuridine), 10 mM: Prepare 5-fluoro-2'-deoxyuridine (F2dU; Sigma-Aldrich, #F0503) as a stock of 10 mM in H_2O. Sterilize by filtration (0.22 µm). Aliquot and store up to 1 year at−20°C.
6. FeSO4, 0.01%: Dissolve 0.05 g of $FeSO_4.7H_2O$ in 500 mL of sterilized H_2O. Sterilize by filtration (0.22 µm). Aliquot and store up to 1 year at −20°C.
7. Geneticin (G418), 100 mg/mL: Purchase ready-to-use solution of 100 mg/ml (Invivogen). Aliquot and store up to 1 year at −20°C.
8. Glucose, 20%: Dissolve 100 g of glucose in 500 mL of H_2O. Sterilize by filtration (0.22 µm). Store up to 1 year at –20°C.
9. Hygromycin B (hygB), 100 mg/mL: Purchase ready-to-use 100 mg/ml hygromycin B solution (Invivogen). Aliquot and store up to 1year at −20°C.
10. IPTG, stock solution, 0.4 M: Prepare isopropyl-β-D-thiogalacto- pyranoside (IPTG; ThermoFisher, #34060) as a stock of 0.4 M in H_2O. Sterilize by filtration (0.22 µm). Aliquot and store up to 1 year at −20°C.
11. Kanamycin, 50 mg/mL: Prepare kanamycin (Roche, #1074 4666) as a stock of 50 mg/ml in H_2O. Sterilize by filtration (0.22 µm). Aliquot and store up to 1 year at −20°C.
12. K- Buffer: Prepare Solution A (K_2HPO_4): Dissolve 200 g of K_2HPO_4 in1 L of H_2O. Autoclave at 120°C for 20 min. Prepare Solution B (KH_2PO_4): Dissolve 145 g of KH_2PO_4 in1 L of H_2O. Autoclave at 120°C for 20 min. Add 150 ml of KH_2PO_4 (Solution B) to 250 ml of K_2HPO_4 (Solution A) to obtain pH 7. Store up to 6 months at 4°C.
13. Luria-Bertani (LB) broth: 10 g/L of tryptone (Pronadisa, #1612.00), 5 g/L of yeast extract (Pronadisa, #1702.00), 10 g/L of NaCl. Store up to 1 month at room temperature.
14. MES, 1 M: Dissolve 63.96 g of MES in 200 ml of sterile H2O. The pH is adjusted to 5.3 with 10 N NaOH with vigorous stirring on a magnetic stirrer until the MES has completely dissolved. Then, H_2O is added to bring the final volume to 300 ml. Sterilize by filtration (0.22 µm). Solution can be aliquoted and frozen for up to 1 year at −20°C.
15. MN solution: Dissolve 30 g of $MgSO_4.7H_2O$ and 15 g of NaCl in 1 L of H_2O. Autoclave at 120°C for 20 min. Store up to 6 months at 4°C.

16. Moxalactam, sodium salt, 100 mg/mL: Prepare moxalactam, sodium salt (Sigma-Aldrich, #M8158) as a stock of 100 mg/ml in H_2O. Sterilize by filtration (0.22 µm). Aliquot and store up to 1 year at −20°C.
17. NH_4NO_3, 20%: Dissolve 20 g of NH_4NO_3 in 100 mL of H_2O. Autoclave at 120°C for 20 min. Store up to 6 months at 4°C.
18. Oligoelements: Dissolve 100 mg of $ZnSO_4.7H_2O$, 100 mg of $CuSO_4.5H_2O$, 100 mg of H_3BO_3, 100 mg of $MnSO_4.H_2O$ and 100 mg of $Na_2MoO_4.2H_2O$ in 1 L of H_2O. Autoclave at 120°C for 20 min. Store up to 6 months at 4°C.
19. Rifampicin, 10 mg/mL: Prepare rifampicin (Sigma-Aldrich, #R3501) as a stock of 10 mg/ml in methanol. Protect from light. Aliquot and store up to 1 year at −20°C.
20. SOC medium: 20 g/L of tryptone (Pronadisa, #1612.00), 5 g/L of yeast extract (Pronadisa, #1702.00), 0.5 g/L NaCl, 0.2 g/L KCl. Adjust to pH 7. Autoclave at 120°C for 20 min, then add: 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose. Store up to 1 month at room temperature.
21. Spectinomycin, 50 mg/mL: Prepare spectinomycin (Fluka, #85555) as a stock of 50 mg/ml in H2O. Sterilize by filtration (0.22 µm). Aliquot and store up to 1 year at −20°C.
22. X-gal stock solution, 20 mg/ml: Prepare 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Fermentas, #R0404) as a stock of 20 mg/ml in dimethylformamide. Aliquot and store up to 1 year at −20°C.

COMMENTARY

Background information

Classical, non-standard restriction-ligation cloning methods using Type II restriction enzymes are inefficient to build multigenic constructs. In principle, precise multigenic constructs could be created by chemical DNA synthesis, which makes tailored DNA sequences without the need of a precursor DNA template. Although DNA synthesis is becoming increasingly affordable, and certainly has become more economical than classical cloning and mutagenesis procedures, it is still cost-prohibitive for multigene-scale DNA synthesis. Moreover, tailored DNA synthesis lacks the advantages of modularity and standardization. Modular design speeds up genetic engineering and decreases the effort required for the assembly of multigenic structures, facilitating the exchange and re-use of assembled parts and multigene modules (Müller & Arndt, 2012).

Type IIS-based and sequence homology-based DNA assembly methods appeared as alternatives to classical cloning with improved efficiency and modularity. Homology-based methods, including USER (Villiers, Stein, & Hollfelder, 2010) and Gibson (Gibson et al., 2009), use overlapping sequences between parts to mediate DNA assembly. These methods enable the scarless assembly of multiple parts in a single reaction. However, a modular approach using these systems implies long scars and sequencing of each newly generated assembly since individual DNA fragments are generated as PCR products. In contrast, type IIS-based restriction-ligation methods rely on a simple one-pot reaction in which parts are assembled in order, leaving relatively small (4 bp) scars between assembled fragments. Type IIS-based methods, including GoldenBraid (GB) and MoClo (Weber, Engler, Gruetzner, Werner, & Marillonnet, 2011), were easy to understand and to adopt by laboratories that were already using classical restriction-ligation in their cloning procedures. A major drawback of these DNA assembly techniques is the requirement for removing internal restriction sites recognized by the restriction enzymes used in each specific system. With GB and

MoClo, hierarchical assembly of multigene constructs can be achieved by the alternate sequential use of two different type IIS restriction enzymes and antibiotic selection markers. MoClo, in contrast to GB, requires a third enzyme for the second level assembly of TUs. Moreover, the gbcloning software offers a straightforward protocol and automates primer design making the domestication or removal of unwanted restriction sites easier. Alternative software tools are available for facilitating MoClo and GB assemblies, including Benchling (https://benchling.com/) and NEB tools (https://goldengate.neb.com/). These tools are suitable for Level 1 or higher level assemblies. However, it should be noted that these tools do not support automatic search and mutation of illegal restriction sites, something that needs to be manually done by the user for each specific sequence prior to start working with them.

Both MoClo and GB rely on the use of Golden Gate for multipartite assemblies at the TU level. However, the solutions they encountered for multigene assemblies are different. While MoClo makes use of multipartite assemblies also at this level, GB uses binary assemblies to grow indefinitely. MoClo offers, therefore, a less time-consuming solution to create multigene structures, but at the expense of flexibility and simplicity. While GB uses a set of only 4 destination vectors (α1, α2, Ω1 and Ω2), the set of vectors required for the assembly of multigene cassettes with MoClo is larger. Moreover, having each TU in a different vector makes its reusability much more limited. Another consideration is the efficiency of the reactions. In general, Golden Gate assemblies have high efficiency, but the efficiency decays with the increment in the number of parts included in the reaction and with the size of those parts. Therefore, Golden Gate multipartite assemblies are highly efficient for assembling TUs from individual parts, but the efficiency is reduced for the assemblies of multigene cassettes from TUs due to the increment in size.

In addition to the advantages stated above, GB is, to our knowledge, the only type IIS-based assembly method that provides an integrated web-based platform available at gbcloning.upv.es for facilitating *in silico* design of multigene constructs. The set of web-based tools was recently expanded to include specific FB DNA parts and structures, such as the gene disruption-cassettes described here.

The most commonly used methods for obtaining fungal transformation constructs (whether inserting or deleting specific genes), require plasmid generation where overlapping sequences are used to allow for the assembly of DNA parts. This results in the disadvantages mentioned above and the necessity to sequence all constructs each time is an additional pitfall.

In some cases, obtaining constructs requires PCR amplification to generate the necessary gene of interest and the selection marker. All of them must be verified by sequencing each time and assembled using a Fusion PCR (Szewczyk et al., 2007), whose product is then integrated into the corresponding binary vector (Garrigues, Gandía, & Marcos, 2016). It is also common in fungi to employ USER methodologies (Salazar-Cerezo, Kun, de Vries, & Garrigues, 2020) or Gibson (Chen, Liu, Duan, Pan, & Liu, 2020) to obtain constructs of interest in fungal molecular biology.

The FB application of the GB methodology greatly simplifies the technique and saves time in the generation of constructions, since, for example, there are universal pieces that can be used in different fungi and once generated and sequenced, they do not have to be sequenced again every time that they are employed in a different construction.

Critical parameters

Domestication process – Mutations

When a CDS has internal illegal BsaI and/or BsmBI sites, synonymous mutations need to be done in order to maintain the open reading frame. This is automatically done at https://gbcloning.upv.es/do/domestication/ for the CDS (B3-B4-B5) category. However, when an illegal site is present in a promoter or in a terminator sequence, the mutation that is automatically introduced at https://gbcloning.upv.es/do/domestication/ is random. Usually a single nucleotide mutation does not affect the performance of the regulatory sequence. However, if the mutation affects for example a critical region of a promoter, expression levels might be affected. Expression assays are recommended in order to assess the performance of newly domesticated regulatory sequences.

Domestication reactions - Considerations

BsmBI and BsaI recognition sites do not occur very often in genomic sequences. However, there are occasions in which two unwanted restriction sites are too close to each other or to the start/end of the sequence. If that occurs small PCR products would be required in order to perform a standard domestication process. However, PCR products smaller than 70 base pairs are difficult to verify on agarose gels. Therefore, for fragments smaller than 70 bp the gbcloning domestication tool recommends the use of long primers to incorporate the intended mutation as more suitable. Alternatively, DNA synthesis can be a more economical alternative if long primers are needed for mutating several proximal internal unwanted sites.

Domestication reactions involving 4+ PCR products or PCR products of substantially different lengths might have low efficiency. In these cases, DNA synthesis also becomes a good alternative to PCR.

DNA amounts in restriction-ligation reactions

Using equimolar amounts of each plasmid is not always required since type IIS-based reactions are highly efficient. However, the use of equimolar amounts of DNA parts is critical for multipartite reactions with 5+ DNA parts. The use of 20 fmoles is recommended for plasmid DNA and 60 fmoles for linear DNA.

Use of the "stuffer-fragment (SF)" plasmids

To assemble TUs/modules of different levels *i.e.* an α1 TU with a Ω2 TU 3use of stuffer fragment plasmids is required. These plasmids facilitate swapping a TU/module from any vector of one level (α or Ω) to any vector of the opposite level (α or Ω). Combining a TU in pDG3α1 with a TU in pDGB3Ω1 would require first either to swap the pDG3α1_TU to pDGB3Ω2 using the pDG3α2_SF or to swap the pDGB3Ω1_TU to pDG3α2 using the pDGB3Ω2_SF. After this first step, the two TUs could be immediately combined. Therefore, one extra cloning step is enough to combine TUs/modules that are assembled in non-compatible plasmids. These plasmids favor the reusability of any TUs/modules previously assembled with GB and are needed for example for 3X TU assemblies.

Storage and handling of T4 ligase buffer

The ATP concentration is critical for the performance of the T4 ligase and therefore for the success of the restriction-ligation reactions. Following manufacturer's indications to minimize degradation of the ATP in the buffer, it is recommended to store it in small aliquots (50 µl) at -20 ºC.

Selection of restriction enzymes for plasmids verification

Restriction enzyme for assembly verification should be selected for each specific plasmid. However, each GB plasmid has sites for a specific restriction enzyme that releases the insert from the backbone. These enzymes are: NotI for pUPD2, EcoRI for pDGBα1 and pDGBΩ1R, HindIII for pDGBα2 and pDGBΩ2R, BamHI for pDGBα1R and pDGBΩ1 and EcoRV for pDGBα2R and pDGBΩ2. The use of these enzymes is recommended for TU/module size verification unless the size of the insert is equal or similar to the size of the vector.

Position of the selection marker in the final assembly for stable transformation

The GB plasmids are designed in a way that the T-DNA left and right borders are flanking the LacZ-cassette and therefore the selection marker TU needs to be included in the assembly as any other TU. Since it is known that the transfer of the T-DNA to the plant / fungi starts on the right border and ends on the left border, we recommend to assemble the selection marker TU next to the left border and in reverse orientation for stable transformation. Either α1R or Ω1R plasmids can be used for this purpose.

Troubleshooting

Plates with many blue colonies

Getting many blue colonies on a plate is indicative of a bad performance of the restriction enzyme. Consider purchasing a new one.

Plates without colonies

No colonies on the plates is indicative of a bad performance of the T4 Ligase. Alternatively, it might indicate that a DNA part is missing on the assembly or that any of the used DNA parts does not have the right overhangs.

Level 1 plasmids lacking one Level 0 part

Although theoretically the T4 ligase is not able to ligate non-compatible overhangs, we occasionally get incorrect assemblies in the laboratory when a “problematic” DNA part is present. The first suggestion in this case is to include higher amounts of that DNA part (60 fmoles instead of 20 fmoles). A second suggestion is to test an equivalent assembly with an alternative promoter / terminator if one of them is the missing part and the use of a specific regulatory region is not crucial for the function of the intended assembly.

Level >1 plasmids lacking one Level 1 TU

Although theoretically the T4 ligase is not able to ligate non-compatible overhangs, we occasionally get wrong assemblies in the laboratory in constructs containing a “problematic” DNA part. The first suggestion in this case is to include higher amounts of that DNA part (60 fmoles instead of 20 fmoles). A second suggestion is to exchange the position of the TUs to be assembled. To do so, cloning them in opposite plasmids (1 instead of 2 and vice versa) is required. Alternatively, the inclusion of stuffer-fragments (SF) in between the two TUs might help to get the intended assembly. This strategy resulted in correct assemblies in our hands when encountering problems with the assembly of TUs facing away one to each other and having the same promoters.

Selection plates with many fungal transformant colonies

Sometimes, selection plates may appear full of colonies. In such cases not everything that is observed are positive transformants, since the background prevents the efficient selection of antibiotics. Plate washes allow to discriminate and select positive clones.

Selection plates without fungal transformant colonies

In the case of lack of growth on the selection plates or in the case of slow growing fungal strains, increase the co-cultivation time from 2 to 4 days.

Selection plates with Agrobacterium colonies

Sometimes selection plates (90 mm) used to spread the washes obtained from previous selection plates (50 mm) may appear with *Agrobacterium* colonies. In that case, supplement the plates with cefotaxime and moxalactam as in previous plates.

Understanding results

Efficiency of the restriction-ligation reactions

Although the efficiency of the transformation depends on the method and on the competent cells used, in general Type IIS-based restriction-ligation reactions are highly efficient for the assembly of up to 5 DNA parts resulting in plates with hundreds of colonies. Most screened white colonies are correct (Sarrion-Perdigones et al., 2011). However, assemblies of 5+ DNA parts or involving parts with complex DNA structures are less efficientand the intended construct cannot be obtained without optimizing the reaction conditions. The use of equimolar DNA amounts in the reactions and increasing the number of cycles of the restriction-ligation protocol from 25 to 50 might help to improve the efficiency.

Plasmid verification

Level 0 parts are verified by a restriction analysis followed by Sanger sequencing of the plasmids found to be correct in the restriction analysis screening. Sanger sequencing is required for Level 0 parts since a PCR amplification step was performed prior to the restriction-ligation reaction. Sequencing of Level 0 parts assembled in the pUPD2 can be done using the following primers pUPD2_Fwd: 5' – GCTTTCGCTAAGGATGATTTCTGG and pUPD2_Rev: 5' – CAGGGTGGTGACACCTTGCC. For verification of higher-level assemblies, a restriction analysis is enough to confirm that all parts/TUs were correctly assembled and that no rearrangements occurred in *E. coli*. Since the reactions from Level 0 to Level 1 and from Level 1 to Level >1 only involve restriction-ligations, no mutations are expected.

Time considerations

As discussed in the **Strategic Planning** section, the assembly of a 2 TU construct takes 10 working days and the whole experiment from primer design to result visualization in fungi would take around one month. The steps of the basic protocols can be summarized as depicted in **Figure 2**.

Acknowledgements

This article is dedicated to the memory of our friend and colleague Dr. Alejandro Sarrion-Perdigones, an early developer of GoldenBraid. We acknowledge the excellent technical assistance provided by Marisol Gascón (IBMCP, Valencia, Spain) with the fluorescent images. This work was funded by Grant BIO2013-42193 and Grant BIO2016-78601-R, Plan Nacional I+D, Spanish Ministry of Economy and Competitiveness, RTI2018-101115-B-C21 from the "Ministerio de Ciencia, Innovación y Universidades" (Spain) (MICINN/FEDER Funds), and PROMETEO/2018/066 from 'Conselleria d'Educació' (Generalitat Valenciana, Comunitat Valenciana, Spain) and SUSPHIRE PCI2018-092893–ERA CoBioTech (109) (MCIU/FEDER).

Conflicts of interest

Authors declare no conflict of interest.

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INTERNET RESOURCES

https://gbcloning.upv.es/

Website for software-assisted GoldenBraid and FungalBraid assemblies. It includes a public database of DNA parts that have been previously adapted to the standard.

https://benchling.com

Highly recommended cloud-based platform for Life Sciences that includes options for vector mapping visualization, restriction analysis design, Type IIS-based cloning or DNA alignments.

FIGURE LEGENDS

Figure 1. GoldenBraid assembly strategy. A) General strategy for Level 0 parts domestication. A DNA sequence having an internal illegal site (BsaI or BsmBI) is domesticated by amplifying the target sequence in two separated PCR products. External GB-adapted primers (GBU-Fwd and GBU-Rev) are designed so they include a BsmBI recognition site, the cleavage sites for cloning into pUPD2, and the 4-nucleotide barcodes (1234) in addition to approximately 20 sequence specific nucleotides. Internal GB-adapted primers (GBI-Fwd and GBI-Rev) have extensions that incorporate the BsmBI recognition sites and the single nucleotide change (M) as part of the BsmBI cleavage site (NNMN) to mutate internal illegal sites. After the amplification of both PCR products, a BsmBI restriction–ligation reaction will bring the domesticated sequence into the pUPD2. B) Simplified representation of the GB grammar for a basic TU. C) Multipartite assembly of a basic Level 1 TU in a pDGB α destination vector. A promoter, CDS and terminator Level 0 parts are assembled together in a pDGB α destination vector with a BsaI reaction. D) Consecutive binary assemblies of 2X TUs in pDGB Ω and 4X TUs in pDGB α with BsmBI and BsaI reactions, respectively.

Figure 2. Timeline of a plant or fungi Agrobacterium-mediated transformation experiment from GoldenBraid assembly to result visualization. GoldenBraid cloning steps (orange section of the arrow) are used to assemble a multigene plasmid that is subsequently transformed in Agrobacterium tumefaciens (yellow section of the arrow) for Agrobacterium-mediated plant or fungi transformation (blue section of the arrow). Several assays are conducted to characterize the obtained plant / fungus transformants (green section of the arrow).

Figure 3. Schematic representation of the software assisted GoldenBraid (GB) assembly process for a two transcriptional unit (TU) expression cassette. On the left, inputs of the corresponding gbcloning tool and GB elements required to perform each assembly step. On the right, output of the corresponding gbcloning tool and GB element obtained upon restriction-ligation reaction. A) Domestication of any DNA sequence to generate a Level 0 GBpart. B) Multipartite assembly of Level 0 GBparts to create a Level 1 transcriptional unit (TU). C) Binary assembly of two TUs to create a gene-expression cassette.

Figure 4. Generation of fluorescently labeled fungal strains. A) Schematic diagram of the multipartite assembly of different TUs and of the binary assembly of vector FB027 (containing the hygromycin resistance (FB003 element) and the element that expresses the yellow fluorescent protein YFP (FB026 element)) and vector FB039 (containing the G418 resistance (FB009 element) and the FB026 element). B) Fungal plates (PDA) after 4 days of growth at 25 ºC and fluorescent images of mycelium and conidiophores (white arrowheads) from representative transformants obtained with the FB027 construct (ANEM001 from *A. niger*; PDMH021 from *P. digitatum* and PEMG041 from *P. expansum*). C) Fungal plate (PDA) after 4 days of growth at 25 ºC and fluorescence microscopy images of mycelium and conidiophores (white arrowheads) from one representative transformant obtained with the FB039 construct (PEMG154 from *P. expansum*).

Figure 5. Scheme of the different steps of the *Agrobacterium tumefaciens*-mediated transformation (ATMT). Different steps of each part of the ATMT protocol from conidia

inoculation to final monosporic culture. In black, flow diagram of ectopic transformation (Basic protocol 2) and in red, flow diagram of the screening process to obtain gene deletion mutants (Basic protocol 4).

Figure 6. Targeting construct design and genetic transformation for *msb2* gene disruption by homologous recombination using a dual selection/counterselection strategy. A) Diagram of the GB grammar used to generate *msb2* disruption construct modified from Hernanz-Koers et al., 2018. FB012 and FB013 elements are universal positive (*hph* for Hyg^R) and negative (HSV*tk* for F2dU) selection markers. B) Schematic representation of the binary assembly of vector FB024 using universal elements and specific elements (FB018 and FB019) for *msb2* disruption. These specific elements are DNA flanking fragments (approximately 1 Kb) to allow homologous recombination. They have to be generated *de novo* for disruption of each gene of interest. C) Scheme for comparison of the *msb2* gene of the parental *P. digitatum* strain CECT 20796 (top); the targeting vector (FB024) designed for gene disruption (middle); and the Δ*msb2* disrupted locus (bottom). All primers used for molecular characterization of transformants are indicated in the figure. C) PCR verification of genomic DNA to verify positive Δ*msb2* transformants (in blue) of *P. digitatum*, with different primer pairs as indicated. Outside primers 566/567, not included in 5' or 3' flanking regions, produced fragment sizes of 4.8 Kb in length from the wt CECT 20796 strain, as well as ectopic transformants (PDMG5342 from this transformation experiment and PDMG612 from a previous transformation experiment) containing a genomic transgene encompassing the msb2 locus inserted in a region different from the targeted locus, or 4.0 kb from positive homologous recombination transformants (PDMG5312, PDMG5328 and PDMG5334). Primers 197/232 revealed the presence of the hygromycin resistance cassette in all transformants. Additional controls included are the plasmid control (FB024) and the negative control (ddH_2O). D) Growth of the parental CECT 20796 and disruption strain PDMG5334 (in blue) on PDA plates after 6 days of incubation at 25 ºC, demonstrating a different phenotype of growth in the Δ*msb2* PDMG5334 strain.

TABLES

Table 1. Examples of GoldenBraid for multigene engineering and gene disruption in different organisms.

Organism	Targeted genome	Type of integration	Examples	Assembly strategy
Plants	Nuclear	Pseudo-random (mediated by Agrobacterium)	Proanthocyanidin biosynthesis in *N. tabacum* by expression of AmRosea1, AmDelila, MtANR and MtLAR (Fresquet-Corrales et al., 2017)	Binary assemblies of the SM and GOI TUs.
	Plastid	Targeted (HR)	Expression of the potential viral entry inhibitor griffithsin in *N. tabacum* plastids (Vafaee et al., 2014)	Subsequent binary assemblies of FS and SM and GOI TUs.
	-	No integration; transient expression (mediated by Agrobacterium)	Recombinant expression of secretory IgA versions in *N. benthamiana* (Juarez et al., 2013); indigo precursors biosynthesis engineering (Frabel et al., 2018)	Binary assemblies of the GOI TUs.
Yeast	Nuclear	Targeted (HR)	Mitochondria targeted expression of the nitrogen fixation *nifU* gene in *S. cerevisiae* (Pérez-González et al., 2017)	Adaptation of the GB backbones for including FS. Binary assembly of the SM and GOI TUs.
Filamentous fungi	Nuclear	Pseudo-random (mediated by Agrobacterium)	YFP expression in *P. digitatum* (Pérez-González et al., 2017)	Binary assembly of the SM and GOI TUs.
		Targeted (HR)	*hog1* gene disruption in *P. digitatum* (Hernanz-Koers et al., 2018)	Multipartite assembly of FS and positive and negative SM TUs.
Human cells	Nuclear	Pseudo-random (cell transfection)	Luciferase-mediated multiple cellular pathways analysis in HEK293T/17 cells (Sarrion-Perdigones et al., 2019)	Subsequent binary assemblies SM and GOI TUs.

HR, homologous recombination; FS, flanking sites; SM, selection marker; GOI, gene of interest; TU, transcriptional unit.

Table 2. List of Level 0 part categories with their associated function and prefix and suffix

Part Category	**Function**	**Prefix**	**Suffix**	**Examples in this protocol**
Fungal PROM+5UTR (A1-A2-A3-B1-B2)	Promoter	GGAG	AATG	p*gpdA*(FB007)
Fungal CDS (B3-B4-B5)	Coding sequence	AATG	GCTT	pYFP(GB0053)
Fungal 3UTR+TERM (B6-C1)	Terminator	GCTT	CGCT	p*trpC*(FB008)
Negative marker (A1-A2-A3; TU domesticated as Level 0 part)	Selection marker	GGAG	TACT	p*gpda*:HSV*tk*:T*tub* (FB013)
Flank 5prime (B1-B2)	5' homology arm	TACT	AATG	p*msb2*_5'FS (FB018)
Positive marker (B3-B4-B5; TU domesticated as Level 0 part)	Selection marker	AATG	GCTT	p*trpC*:*hph*:T*tub* (FB012)
Flank 3prime (B6-C1)	3' homology arm	GCTT	CGCT	p*msb2*_3'FS (FB019)

TU; transcriptional unit

Table 3. Primers for fungal promoter domestication to the GoldenBraid grammar

Primer	Sequence (5'→ 3')
GB Universal forward primer (Fwd)	nnnn*CGTCTC*nCTCGGGAG(~20 first sequence specific nts)
P*gpdA* Fwd	GCGC*CGTCTC*GCTCGGGAGTGGCGCATGCGGACAGACGG
GB Universal reverse primer (Rev)	nnnn*CGTCTC*nCTCACATT(~20 last sequence specific nts)
P*gpdA* Rev	GCGC*CGTCTC*GCTCACATTTTAAAGGTTCTTGGATGGGAAG

In italics BsmBI recognition site. Underlined pUPD2 overhangs. In blue, part specific overhangs.

Table 4: Stock solution and transformation media

	Stock Solution (100 mL)[a]			MM	IM	CM[b]
Reagent	Chemical	Amount required	Sterilized			
Sterile H_2O			120 ºC 20 min	93.5 mL	88.5 mL	88.5 mL
K buffer (pH 7.0)	K_2HPO_4 KH_2PO_4	20 g 14.5 g		1 mL	1 mL	1 mL
50% Glycerol	Glycerol	50 mL	120 ºC 20 min		1 mL	1 mL
Agar			120 ºC 20 min			1.5 g
MN Solution	$MgSO_4.7H_2O$ NaCl	3 g 1.5 g	120 ºC 20 min	2 mL	2 mL	2 mL
1% $CaCl_2$	$CaCl_2.2H_2O$	1 g	120 ºC 20 min	0.1 mL	0.1 mL	0.1 mL
Oligoelements	$ZnSO_4.7H_2O$ $CuSO_4.5H_2O$ H_3BO_3 $MnSO_4.H_2O$ $Na_2MoO_4.2H_2O$	0.01 g 0.01 g 0.01 g 0.01 g 0.01 g	120 ºC 20 min	1 mL	1 mL	1 mL
20% NH_4NO_3	NH_4NO_3	20 g	120 ºC 20 min	0.25 mL	0.25 mL	0.25 mL
20% Glucose	Glucose	20 g	Filtration (0.22)	1 mL	1 mL	1 mL
0.01% $FeSO_4$	$FeSO_4$	0.01 g	Filtration (0.22)	1 mL	1 mL	1 mL
1M MES (pH 5.3)	MES	21.32 g	Filtration (0.22)		4 mL	4 mL
Antibiotic stock solution[e] Kanamycin(K) Spectinomycin (S) (50 mg/mL)			Filtration (0.22)	0.15 mL (K) 0.2 mL (S)	0.15 mL (K) 0.2 mL (S)	0.15 mL (K) 0.2 mL (S)
Acetosyringone 200 mM					0.2 mL	0.2 mL

[a] Store them at 4°C except Glucose, FeSO4, antibiotics, acetosyringone, and MES (stored at −20°C).

[b] Similar to IM medium except the addition of 1.5% agar.

[e] Antibiotic depends of binary plasmid used to transform. Kanamycin for pDGB3α and spectinomycin for pDGB3Ω

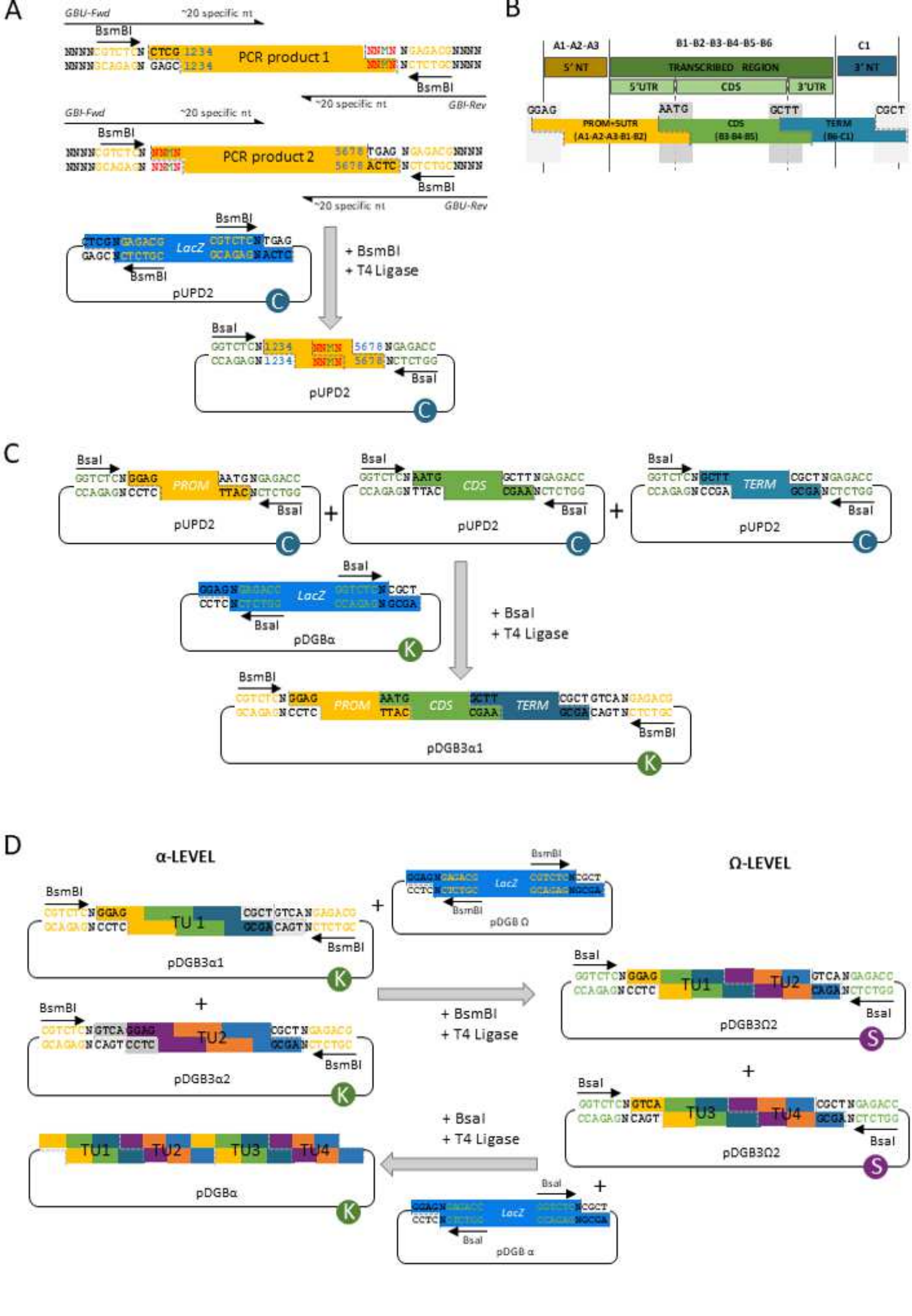
Table 5. Primers for flanking site domestication to the GoldenBraid grammar

Primer	Number	Sequence (5'→ 3')
General forward primer (5'FS)		nnnn*CGTCTC*nCTCGTACT(~20 first sequence specific nts)
msb2 5'FS Fwd.	547 Fwd	GCGC*CGTCTC*GCTCGTACTGAAGGTTGATTTATAATCCCC TTC
General reverse primer (5'FS)		nnnn*CGTCTC*nCTCACATT(~20 last sequence specific nts)
msb2 5'FS Rev.	548 Rev	GCGC*CGTCTC*GCTCACATTAAAAAACAAATTGACAATAA/ CGAATGAA
General forward primer (3'FS)		nnnn*CGTCTC*nCTCGGCTT(~20 first sequence specific nts)
msb2 3'FS Fwd.	549 Fwd	GCGC*CGTCTC*GCTCGGCTTACCCCACAATTCCCCTCGAG
General reverse primer (3'FS)		nnnn*CGTCTC*nCTCAAGCG(~20 last sequence specific nts)
msb2 3'FS Rev.	550 Fwd	GCGC*CGTCTC*GCTCAAGCGGGGGGGGAGGGGGGAAACC AT

In italics BsmBI recognition site. Underlined pUPD2 overhangs. In blue, part specific overhangs.

Table 6. Primers used for verification of fungal transformant strains

Primer	Sequence (5'→ 3')	Use
197 forward primer	CGTTAACTGATATTGAAGGAGCAT	Verification of hygromicin resistance cassette (FB012 element)
232 reverse primer	GTTTGCCAGTGATACACATGGG	Verification of hygromicin resistance cassette (FB012 element)
311 forward primer	CCACGGAAGTCCGCCCGGAGC	Verification of HSV*tk* gene (FB013 element)
312 reverse primer	GACGTGCATGGAACGGAGGCG	Verification of HSV*tk* gene (FB013 element)
566 forward primer	GTCTATACTTCGGACTCCGC	Primer located outside *msb2* locus to discriminate parental and disruption transformants
567 reverse primer	CAAGGCCATCTGGCACTTGGGC	Primer located outside *msb2* locus to discriminate parental and disruption transformants


A
GBU-Fwd
~20 specific nt
BsmBI
PCR product 1
BsmBI
~20 specific nt
GBI-Rev
GBI-Fwd
~20 specific nt
BsmBI
PCR product 2
BsmBI
~20 specific nt
GBU-Rev
BsmBI
LacZ
BsmBI
pUPD2
+ BsmBI
+ T4 Ligase
BsaI
BsaI
pUPD2
B
A1-A2-A3
B1-B2-B3-B4-B5-B6
C1
5' NT
TRANSCRIBED REGION
3' NT
5'UTR
CDS
3'UTR
GGAG
AATG
GCTT
CGCT
PROM+5UTR
(A1-A2-A3-B1-B2)
CDS
(B3-B4-B5)
TERM
(B6-C1)
C
BsaI
PROM
BsaI
pUPD2
BsaI
CDS
BsaI
pUPD2
BsaI
TERM
BsaI
pUPD2
BsaI
LacZ
BsaI
pDGBα
+ BsaI
+ T4 Ligase
BsmBI
PROM
CDS
TERM
BsmBI
pDGB3α1
D
α-LEVEL
Ω-LEVEL
BsmBI
TU 1
BsmBI
pDGB3α1
BsmBI
LacZ
BsmBI
pDGB Ω
BsmBI
TU2
BsmBI
pDGB3α2
+ BsmBI
+ T4 Ligase
BsaI
TU1
TU2
BsaI
pDGB3Ω2
BsaI
TU3
TU4
BsaI
pDGB3Ω2
+ BsaI
+ T4 Ligase
TU1
TU2
TU3
TU4
pDGBα
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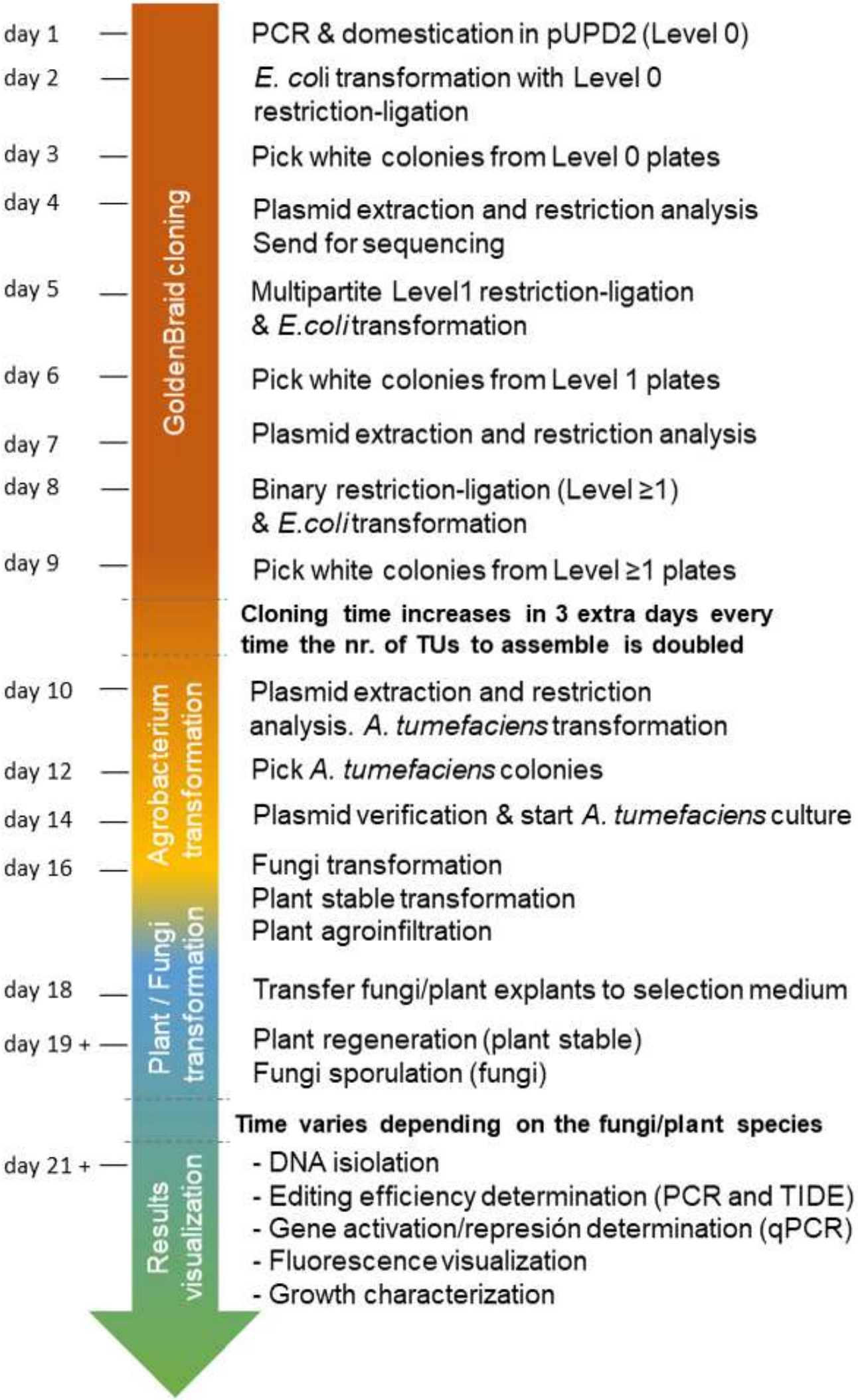
LacZ
BsaI
pDGB α


day 1
day 2
day 3
day 4
day 5
day 6
day 7
day 8
day 9
day 10
day 12
day 14
day 16
day 18
day 19 +
day 21 +
GoldenBraid cloning
Agrobacterium transformation
Plant / Fungi transformation
Results visualization
PCR & domestication in pUPD2 (Level 0)
E. coli transformation with Level 0 restriction-ligation
Pick white colonies from Level 0 plates
Plasmid extraction and restriction analysis
Send for sequencing
Multipartite Level1 restriction-ligation & E.coli transformation
Pick white colonies from Level 1 plates
Plasmid extraction and restriction analysis
Binary restriction-ligation (Level ≥1) & E.coli transformation
Pick white colonies from Level ≥1 plates
Cloning time increases in 3 extra days every time the nr. of TUs to assemble is doubled
Plasmid extraction and restriction analysis. A. tumefaciens transformation
Pick A. tumefaciens colonies
Plasmid verification & start A. tumefaciens culture
Fungi transformation
Plant stable transformation
Plant agroinfiltration
Transfer fungi/plant explants to selection medium
Plant regeneration (plant stable)
Fungi sporulation (fungi)
Time varies depending on the fungi/plant species
- DNA isiolation
- Editing efficiency determination (PCR and TIDE)
- Gene activation/represión determination (qPCR)
- Fluorescence visualization
- Growth characterization

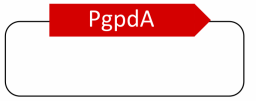
A

DOMESTICATION REACTION

Level 0 GBparts

https://gbcloning.upv.es/fungal/do/domestication

PCR amplification



gpdA promoter (pUPD2)

Category:fungal_PROM+5UTR (A1-A2-A3-B1-B2)

Prefix:GGAG

Suffix:AATG

You can download a detailed protocol to fulfill the assembly in the lab from here

You can download your new seq in genbank format here

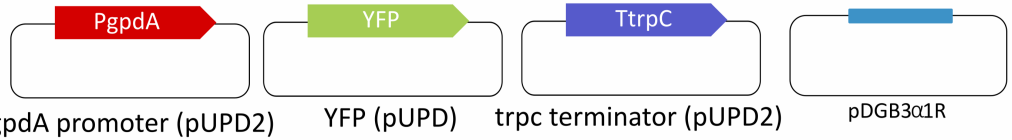
You can download your new seq in SBOL format here

+Add part

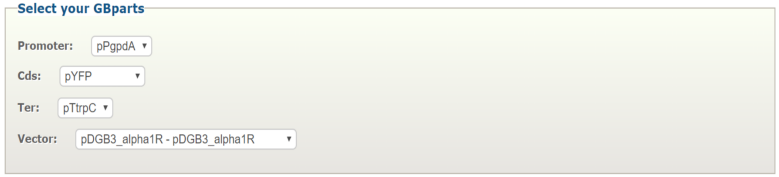
B

MULTIPARTITE ASSEMBLY

Level 1 GBtranscriptional units



gpdA promoter (pUPD2) YFP (pUPD) trpc terminator (pUPD2)



https://gbcloning.upv.es/fungal/do/multipartite

TU-YFP (pDGB3α1R)

You have assembled in the GoldenBraid destiny vector pDGB3_alpha1R the following parts:

Fungal 3UTR+TERM (B6-C1):FB008

Fungal CDS (B3-B4-B5):GB0053

Fungal PROM+5UTR (A1-A2-A3-B1-B2):pPgpdA

You can download a detailed protocol to fulfill the assembly in the lab from here

The resulted sequence of the assembly is genbank_file

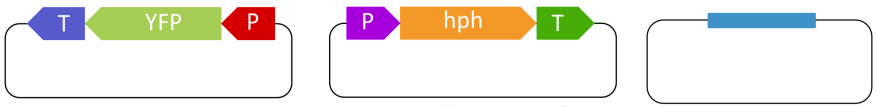
The resulted sequence of the assembly is SBOL_file

+Add part

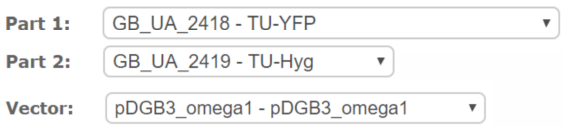
C

BINARY ASSEMBLY

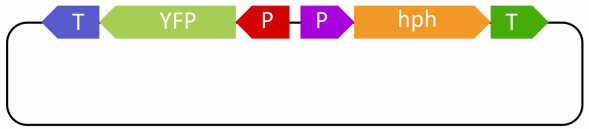
Level >1 GBmodules



TU-YFP (pDGB3α1R) TU-hph (pDGB3α2)



https://gbcloning.upv.es/fungal/do/binary



TU-YFP-TU-hph (pDGB3Ω1)

You have assembled in the GoldenBraid destiny Vector pDGB3_omega1 the following parts:

Part 1:GB_UA_2418

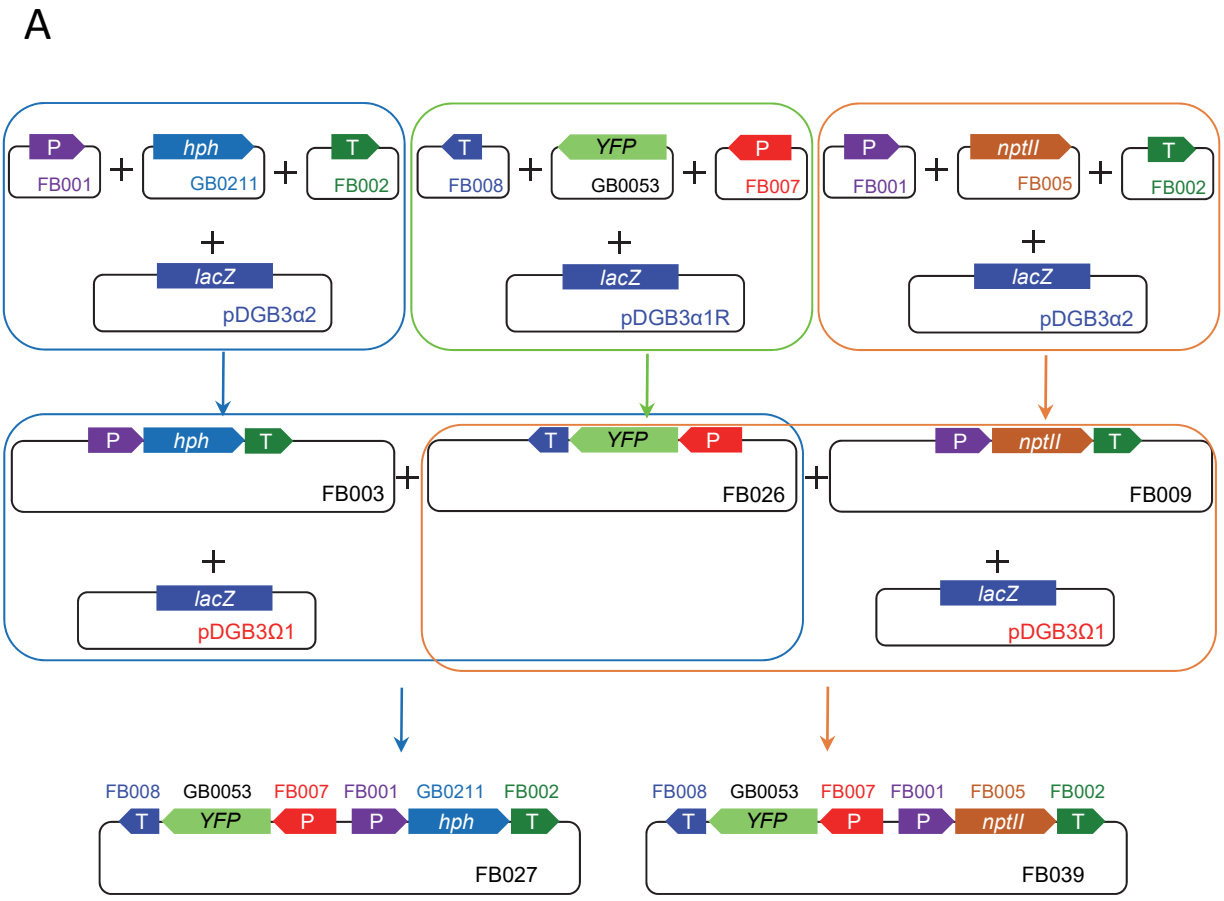
Part 2:GB_UA_2419

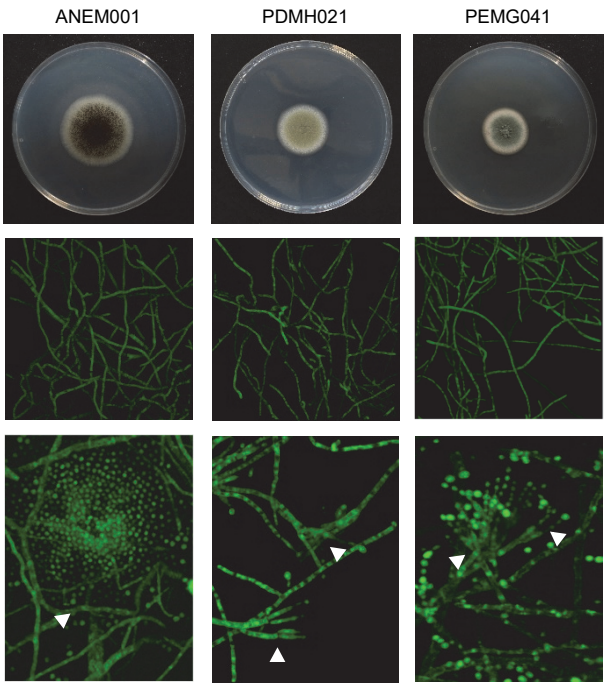
You can download a detailed protocol to fulfill the assembly in the lab from here

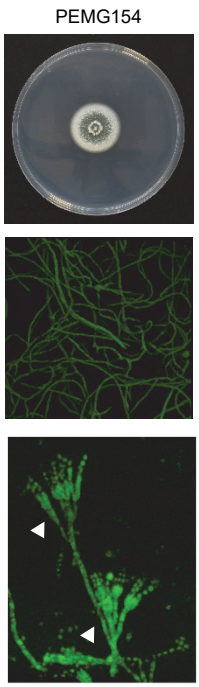
The resulted sequence of the assembly is genbank_file

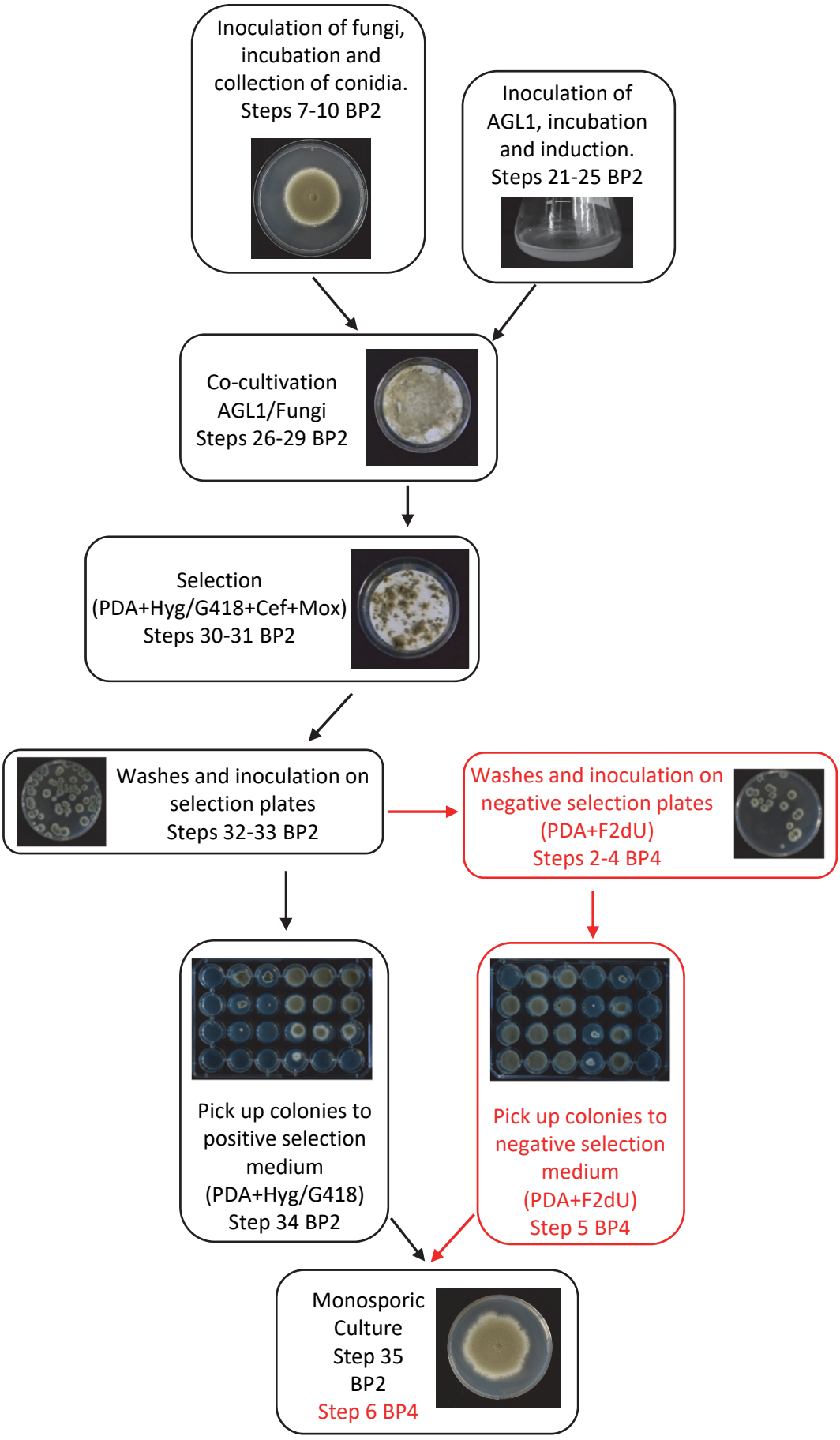
The resulted sequence of the assembly is SBOL_file

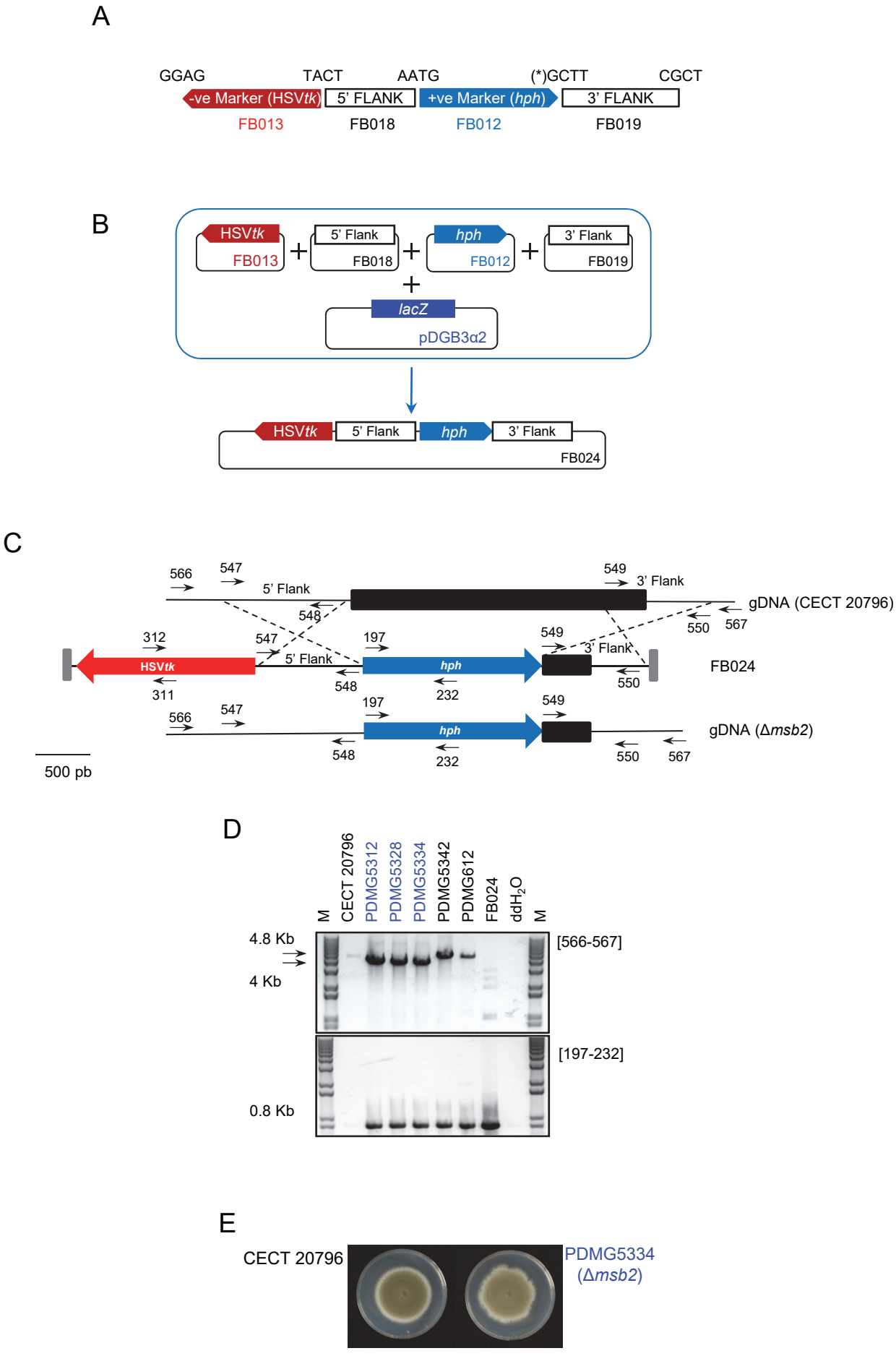
+Add part


A
P
FB001
hph
GB0211
T
FB002
lacZ
pDGB3α2
T
FB008
YFP
GB0053
P
FB007
lacZ
pDGB3α1R
P
FB001
nptII
FB005
T
FB002
lacZ
pDGB3α2
P
hph
T
FB003
lacZ
pDGB3Ω1
T
YFP
P
FB026
P
nptII
T
FB009
lacZ
pDGB3Ω1
FB008 GB0053 FB007 FB001 GB0211 FB002
T YFP P P hph T
FB027
FB008 GB0053 FB007 FB001 FB005 FB002
T YFP P P nptII T
FB039


B
ANEM001
PDMH021
PEMG041


C
PEMG154


Inoculation of fungi, incubation and collection of conidia. Steps 7-10 BP2
Inoculation of AGL1, incubation and induction. Steps 21-25 BP2
Co-cultivation AGL1/Fungi Steps 26-29 BP2
Selection (PDA+Hyg/G418+Cef+Mox) Steps 30-31 BP2
Washes and inoculation on selection plates Steps 32-33 BP2
Washes and inoculation on negative selection plates (PDA+F2dU) Steps 2-4 BP4
Pick up colonies to positive selection medium (PDA+Hyg/G418) Step 34 BP2
Pick up colonies to negative selection medium (PDA+F2dU) Step 5 BP4
Monosporic Culture Step 35 BP2 Step 6 BP4


A
GGAG
TACT
AATG
(*)GCTT
CGCT
-ve Marker (HSVtk)
5' FLANK
+ve Marker (hph)
3' FLANK
FB013
FB018
FB012
FB019
B
HSVtk
FB013
5' Flank
FB018
hph
FB012
3' Flank
FB019
lacZ
pDGB3α2
HSVtk
5' Flank
hph
3' Flank
FB024
C
566
547
5' Flank
549
3' Flank
gDNA (CECT 20796)
548
550
567
312
547
197
549
HSVtk
5' Flank
hph
3' Flank
FB024
311
548
232
550
566
547
197
549
hph
gDNA (Δmsb2)
548
232
550
567
500 pb
D
M
CECT 20796
PDMG5312
PDMG5328
PDMG5334
PDMG5342
PDMG612
FB024
ddH2O
M
4.8 Kb
4 Kb
[566-567]
[197-232]
0.8 Kb
E
CECT 20796
PDMG5334
(Δmsb2)