FungalBraid: a GoldenBraid-based modular cloning platform for the assembly and exchange of DNA elements tailored to fungal synthetic biology.

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Abstract

Current challenges in the study and biotechnological exploitation of filamentous fungi are the optimization of DNA cloning and fungal genetic transformation beyond model fungi, the open exchange of ready-to-use and standardized genetic elements among the research community, and the availability of universal synthetic biology tools and rules. The GoldenBraid (GB) cloning framework is a Golden Gate-based DNA cloning system developed for plant synthetic biology through Agrobacterium tumefaciens-mediated genetic transformation (ATMT). In this study, we develop reagents for the adaptation of GB version 3.0 from plants to filamentous fungi through: (i) the expansion of the GB toolbox with the domestication of fungal-specific genetic elements; (ii) the design of fungal-specific GB structures; and (iii) the ATMT and gene disruption of the plant pathogen Penicillium digitatum as a proof of concept. Genetic elements domesticated into the GB entry vector pUPD2 include promoters, positive and negative selection markers and terminators. Interestingly, some GB elements can be directly exchanged between plants and fungi, as demonstrated with the marker hph for HygR or the fluorescent protein reporter YFP. The iterative modular assembly of elements generates an endless number of diverse transcriptional units and other higher order combinations in the pDGB3α/pDGB3Ω destination vectors. Furthermore, the original plant GB syntax was adapted here to incorporate specific GB structures for gene disruption through homologous recombination and dual selection. We therefore have successfully adapted the GB technology for the ATMT of fungi. We propose the name of FungalBraid (FB) for this new branch of the GB technology
that provides open, exchangeable and collaborative resources to the fungal research community.

**Keywords:** DNA cloning; DNA assembly; Golden Gate; *Penicillium digitatum*; Postharvest pathology; Synthetic biology.
1. Introduction

Fungi are a major threat to human health, crop and animal production and food security (Brown et al., 2012; Fisher et al., 2012). From the beneficial side, some fungal species serve as eukaryotic models in fundamental and applied research (Perez-Nadales et al., 2014). Fungi have also been widely used for the biotechnological production of enzymes and other proteins and a variety of primary and secondary metabolites including organic acids, pigments, antibiotics, hormones and other pharmaceutical compounds (Meyer et al., 2016a). They likewise hold great promise as organisms to be used in synthetic biology to engineer and control the production of existing and novel compounds with commercial applications (Paddon and Keasling, 2014; Mattern et al., 2015; Boecker et al., 2016).

Currently, the limited availability of synthetic biology tools suited to filamentous fungi has been identified as a major obstacle to accelerate the development of fungal biotechnology (Meyer et al., 2016a; Meyer et al., 2016b). The ultimate goal of synthetic biology is to reach biotechnological objectives with the application of principles of engineering disciplines, such as implementation of common standard pieces, designs and construction rules (Kahl and Endy, 2013; Paddon and Keasling, 2014). Plant synthetic biology has been fueled in recent years by the use of highly efficient and high-throughput modular cloning methods based on type IIS restriction enzymes, such as Golden Gate (Engler et al., 2008; Engler et al., 2009), MoClo (Weber et al., 2011) or GoldenBraid (GB) (Sarrion-Perdigones et al., 2011). GB makes use of open-source pCAMBIA-derived vectors for the *Agrobacterium tumefaciens*-mediated genetic transformation (ATMT) of plants (Sarrion-Perdigones et al.,
2013), and its main advantage is the full reusability of its multigenic parts, which results from its iterative cloning strategy. Furthermore, GB cloning is supported by a number of open software tools hosted at a dedicated web page (www.gbcloning.org) that includes a database of standardized parts, as well as documentation tools (Vazquez-Vilar et al., 2017).

These type IIS-based methods have expedited the assembly of multigenic constructs for plant transformation and, most importantly, enabled the exchange of standard DNA parts among plant biotechnologists. Following the initial development of these different systems, the first DNA assembly standard was proposed for plant synthetic biology and includes a common genetic syntax that facilitates the exchange of standard DNA parts (Phytobricks) among laboratories (Patron et al., 2015). The Phytobrick standard was initially agreed upon by representatives of twenty-seven institutions belonging to the international plant science and synthetic biology communities, including inventors, developers and users of type IIS cloning methods. Currently, Phytobrick is a widely used standard among plant synthetic biologists.

Recently, GB has been adapted for the heterologous expression of proteins in yeast mitochondria (Pérez-González et al., 2017). The use of Golden Gate type IIS assembly systems was previously demonstrated for the genetic transformation of filamentous fungi, obtaining gene disruption mutants in the well-studied basidiomycete Ustilago maydis and the ascomycete Aspergillus nidulans fungal models by means of protoplast transformation (Terfrüchte et al., 2014). These previous reports exploited the many advantages of modular genetic elements and their assemblies but, as described, are restricted to fungal organisms for which these transformation procedures and specific plasmids are
suitable, and thus cannot be extended to other fungi. Nowadays, it is well known that ATMT enables the gene disruption and genetic transformation of an increasingly wide number of fungal species by using virtually the same protocol, including non-model fungi for which molecular genetic tools are not well developed (de Groot et al., 1998; Michielse et al., 2005; Frandsen, 2011).

Moreover, the binary plasmids and *Agrobacterium* strains used for ATMT of fungi are fairly standard, and common to those used in plant transformation. In this study, we take advantage of these facts to implement and demonstrate the feasibility of the GB and PhytoBrick standards for the ATMT of filamentous fungi, and the exchangeability of the system between plants and fungi.

Penicillium digitatum is a specific postharvest pathogen of citrus fruit that causes the green mold citrus decay, producing very important economic losses in one of the main fruit tree crops worldwide (Palou, 2014). The main method to control this and other postharvest pathogens is the massive use of chemical fungicides, which causes the appearance of resistance (Holmes and Eckert, 1999) and puts human health and the environment at risk. Molecular and genetic studies have been recently initiated to characterize the *P. digitatum* biology and search for novel control strategies. These include the sequencing of the genome of different *P. digitatum* strains (Marcet-Houben et al., 2012; Sun et al., 2013), which has accelerated the use of reverse genetic approaches to study the biological role and function of specific genes related to virulence and pathogenesis (Zhang et al., 2013a; Zhang et al., 2013b; Gandía et al., 2014; Wang et al., 2014; Harries et al., 2015; Gandía et al., 2016; Garrigues et al., 2016; Ma et al., 2016; de Ramón-Carbonell and Sánchez-Torres, 2017). We have chosen this important plant pathogen to demonstrate the advantages of
using the GB modular cloning standards in non-model, economically important fungi.

2. Materials and methods

2.1. Microorganisms, media and culture conditions

The *P. digitatum* strain CECT 20796 (isolate PHI26) (Marcet-Houben et al., 2012) was used as the fungal parental isolate for transformation. This strain and all transformants generated in this work were cultured on potato dextrose agar (PDA) (Difco 213400) plates for 7-10 days at 25 °C. For growth analyses, 5 µL of conidial suspension (1 x 10^3 conidia/mL) of each *P. digitatum* strain were deposited on the centre of PDA plates and the diameter of growth was measured daily from 3 to 10 days. Vectors generated with different parts were amplified in *Escherichia coli* JM109 grown in Luria Bertani (LB) medium supplemented with either 25 µg/mL chloramphenicol, 50 µg/mL kanamycin or 100 µg/mL spectinomycin at 37 °C depending on the vector. The *A. tumefaciens* AGL-1 strain was grown in LB medium supplemented with 20 µg/mL rifampicin at 28 °C.

2.2. Modular, multipartite and binary assembly reactions to obtain different plasmids

In all assemblies, restriction-ligation reactions were carried out following previously described GB protocols (Sarrion-Perdigones et al., 2011; Sarrion-Perdigones et al., 2013). The GB entry vector pUPD2 and the eight destination vectors from the most recent GB version 3.0 were used (Vazquez-Vilar et al., 2017). Different plasmids and primers used in this work are listed in Tables 1 and 2, respectively. Genetic elements (Table 1) were either amplified through
PCR carried out with AccuPrime High Fidelity polymerase (Invitrogen 12346-086) and specific primers containing 5'/3' DNA barcodes, or provided by an external company (IDT, Integrated DNA Technologies) as synthetic genes (gBlocks gene fragments) (Table 3), in both cases designed according to GB rules and tools (https://gbcloning.upv.es/). Each single genetic element was ligated into the domestication entry vector (pUPD2) through restriction-ligation reactions as described (Sarrion-Perdigones et al., 2013). Positive clones were confirmed by PCR amplification using external primers OJM524 and OJM525 designed for pUPD2 vectors (Table 2). All the resulting DNA constructs were verified by Sanger DNA sequencing and deposited in the GB database.

Single genetic elements cloned into pUPD2 are described in Table 1. The previous GB plasmids and parts are indicated as GB (for instance, GB0211 is the coding sequence of the hygromycin resistance marker) while plasmids produced in this work are noted as FB (for instance FB001 is the promoter from the trpC gene from A. nidulans). All the multipartite and binary assemblies appear in Table 1 in the “Assembly” column. For instance, FB003 is a transcriptional unit (TU) composed of FB001, GB0211 and FB002 modular units, which were assembled into the binary vector pDGB3α2 to drive the expression of the hygromycin resistance gene in filamentous fungi. Similarly, FB009 contains the gene conferring resistance to G418 just by exchanging the coding sequence module (FB005 instead of GB0211). The FB010 plasmid was constructed with the same modular units used in FB003, but assembled into the binary vector pDGB3α1 instead of pDGB3α2 to facilitate a different arrangement in subsequent assemblies. The simplest binary assembly used in this work was FB027 that combines FB003 and FB026 to express the yellow
fluorescent protein (YFP) marker using the hygromycin resistance gene as the fungal selection marker.

In order to generate specific gene constructs to compare the efficiency of Ppaf and PgpdA promoters in the production and secretion of the antifungal protein AfpB from *P. digitatum*, two different multipartite assemblies (FB036/FB037) were generated. These contained the TU for the expression of the *afpB* coding sequence (FB031) and the Tpaf terminator (FB030), under the control of either the *Ppaf* (FB029) or *PgpdA* (FB007) promoters. Plasmids FB029, FB030, FB031 were obtained by direct cloning of synthetic gene fragments into pUPD2, which allowed gene domestication in a simple and inexpensive way.

To generate the specific construct to disrupt the *P. digitatum hog1* gene by homologous recombination and dual selection, multipartite assembly of four single modular units (FB012, FB013, FB014 and FB015) was performed to obtain the binary vector FB022 in a pDGB3α2 backbone. FB012 was obtained using FB003 as a template to amplify the TU encoding for Hyg<sup>R</sup> with OJM535 and OJM536 primers with specific barcodes, and used as the positive selection marker. FB013 was obtained from FB006 with OJM537 and OJM538 primers adapting the GB grammar to allow its reverse orientation (Table 1) and used as negative selection marker. FB014 and FB015 contained two 1 Kb DNA fragments upstream and downstream of the *hog1* coding sequence, and obtained with primers OJM539 and OJM540 (FB014) or with OJM541 and OM542 (FB015).

Positive clones and correct assembly were confirmed by restriction analyses and/or by PCR analyses using combinations of the universal primers
OJM533 and OJM534 specific for pDGB3 vectors, OJM197 and OJM232 for the positive selection marker, and OJM311 and OJM312 for the negative selection marker (Table 2). Further details of other assemblies are shown in the results section and figures below. All binary vectors generated that would later be used for fungal transformation, were introduced into *A. tumefaciens* AGL-1 strain by electroporation.

### 2.3. Fungal transformation

*P. digitatum* strain CECT 20796 was transformed through ATMT essentially as described previously (Khang et al., 2006; Harries et al., 2015). For ectopic transformation, transformed strains were selected in 25 µg/mL hygromycin B (Invivogen, ant-hm-5) or 25 µg/mL geneticin (G418) (Invivogen, ant-gn-5). For homologous recombination, null strains were initially pre-screened in 25 µg/mL hygromycin B as a positive selection and then in 25 µM 5-fluoro-2'-deoxyuridine (F2dU) (Sigma-Aldrich F0503) as a negative selection. Transformants were confirmed by PCR amplification of genomic DNA as described previously (Gandía et al., 2014). The size and presence of DNA amplicons were determined by gel electrophoresis.

### 2.4. Fluorescence microscopy of YFP-tagged transformants

For mycelium visualization, suspensions of 2.5 x 10⁴ conidia/mL of *P. digitatum* strains were grown for 48 h at 25 ºC in 5% PDB medium containing 0.01% chloramphenicol to avoid bacterial contamination. After this, the mycelium was washed, extended and suspended in 20% glycerol. For visualization of the fluorescence in spores, 1 x 10⁶ conidia/mL suspensions of
different strains were used. The YFP fluorescence was visualized in a Nikon E90i fluorescence microscope with excitation/emission wavelengths of 488/535 nm, respectively. The images were captured with a 40X objective using the software NIS-Elements BR v2.3 (Nikon).

2.5. Determination of AfpB production

To verify AfpB protein production, 5.5 x 10^5 conidia/mL of each transformed strain were cultured in liquid *P. digitatum* minimal medium (PdMM) (Sonderegger et al., 2016) for 10 days at 25 ºC with strong aeration. Cell-free supernatants of *P. digitatum* strains were collected and 10 µL of 10-fold concentrated total proteins from the supernatants of each strain were separated by SDS-PAGE electrophoresis using SDS-16 % polyacrylamide gels calibrated with prestained protein size-standard SeeBlue® (ThermoFischer Scientific) and visualized by Coomassie blue staining.

2.6. Sensitivity of null Δhog1 mutants

To confirm the identity of the Δhog1 null strains, their sensitivity to different compounds was tested. Growth in PDA medium supplemented with 1.2 M sorbitol as osmotic stabilizer or 0.5 M NaCl was observed after incubating the strains on 24-well plates at 25 ºC for 4 days. Five µL of serial 10-fold dilutions of conidia (from 1 x 10^5 to 1 x 10^3 conidia/mL) were added to each well.
3. Results and Discussion

3.1. The GoldenBraid cloning strategy, grammar and modular assembly

GB is a Golden Gate-based cloning and DNA assembly strategy that was previously designed for the collaborative development of plant synthetic biology tools (Sarrion-Perdigones et al., 2011). This section summarizes the main features of the GB system. Complete information can be found on the web page (https://gbcloning.upv.es/) and in previous publications (Sarrion-Perdigones et al., 2011; Sarrion-Perdigones et al., 2013; Sarrion-Perdigones et al., 2014; Vazquez-Vilar et al., 2017). Similar to Golden Gate, GB uses type IIS restriction enzymes that cut outside their recognition sequence and generate customized four-nucleotide sticky ends (Engler et al., 2008; Engler et al., 2009). Using the appropriate design principles, subsequent ligation with compatible four-nucleotide overhangs allows for the highly efficient and ordered assembly of as many as 10 DNA fragments in a single tube reaction.

The GB cloning and assembly procedure consists of three separate steps (Fig. 1): Domestication, multipartite assembly and binary assembly. First, a “domesticated” DNA part is engineered to eliminate the internal IIS sites of \( BsmBI \), \( Bsai \) and \( BtgZI \), and to incorporate flanking four-nucleotide barcodes and \( BsmBI \) sites at both ends (Fig. 1A and Supplementary Fig. S1). Domestication is achieved by synthetic gene synthesis or through a PCR procedure that incorporates the nucleotide barcodes with the aid of customized oligonucleotide primers. This DNA part is initially cloned into the pUPD2 entry vector (level 0) in a so-called restriction-ligation reaction containing both \( BsmBI \) and T4 DNA ligase (Supplementary Fig. S1). Any DNA fragment can then be stored in this plasmid as part of the universal library of “DNA parts” (genetic
elements). Second, DNA parts can be excised from the pUPD2 backbone by

*Bsal* digestion (Supplementary Fig. S1) and assembled in order into any of the
four alpha destination plasmids (into pDGB3α1 in the example of Fig. 1B; see
also the cloning site of pDGB3α1 in Supplementary Fig. S2). This step is called
“multipartite assembly” and allows for the barcode-directed assembly of TUs by
combining promoters, terminators, coding sequences, parts that encode signal
peptides, tags or protein in-frame fusions, for example. Third, from here on, an
endless and customized number of “binary assembly” steps can be designed
that allow the assembly –each time- of two α (or Ω) vectors into a single Ω (or α)
vector, alternatively, with the use of *Bsm*BI (*Bsal*) enzymes, producing a
continuous intertwined “braid” of gene constructs (Fig. 1C). There are eight
destination vectors in GB v3.0 (pDGB3): four for each of the α or Ω subsets,
coded as 1, 1R, 2 and 2R (Supplementary Fig. S2). The four different options
(1, 1R, 2 and 2R) of each α/Ω vector permit the desired order and orientation of
TUs. The additional four-nucleotide barcodes incorporated in the eight
destination vectors (color-coded in Supplementary Fig. S2) and the alternate
use of *Bsm*BI and *Bsal* enzymes allow for the endless intertwined steps.
Importantly, the twelve four-nucleotide fusion sites (or barcodes) that flank
each standard DNA part and their order proposed in GB (Fig. 2) have been
adopted by leading groups of plant synthetic biologists as a common standard
and syntax for the exchange of DNA parts (Patron et al., 2015).

3.2. Domestication and assembly of fungal-specific GB elements

In this study, we initiated the construction of a standardized library of
fungal genetic elements using GB standards (Table 1). All the plasmids
constructed in this work will be deposited in public plasmid repositories (i.e.,
Addgene, https://www.addgene.org/). Genetic elements amplified and domesticated by the PCR approach with the aid of specifically designed primers (see Table 2) were: the P\textsubscript{trpC} promoter from \textit{A. nidulans} (Hamer and Timberlake, 1987) amplified from pBHt2 (Mullins et al., 2001); a short intronless version of the P\textsubscript{gpdA} promoter (Punt et al., 1990) and the T\textsubscript{trpC} terminator (Hamer and Timberlake, 1987) from \textit{A. nidulans}, both amplified from pBARGPE1 (Pall and Brunelli, 1993); the T\textsubscript{tub} terminator from \textit{N. crassa} amplified from pGKO2 (Khang et al., 2005); the positive selection marker \textit{nptII} for kanamycin/G418 resistance from \textit{E. coli} (Mazodier et al., 1985) amplified from GB0034 (https://gbcloning.upv.es/); and the complete TU of the negative marker HSV\textit{tk} for F2dU\textsuperscript{S} that includes the P\textsubscript{gpdA} promoter from \textit{Cochliobolus heterostrophus}, the CDS for thiamine kinase (\textit{tk}) from Herpes simplex virus (HSV), and the T\textsubscript{tub} terminator from \textit{N. crassa} amplified from pGKO2 (Khang et al., 2005). Single band amplicons were confirmed by gel electrophoresis before cloning into pUPD2.

Three additional genetic elements were domesticated as synthetic DNAs (Table 3): the P\textsubscript{paf} promoter and T\textsubscript{paf} terminator from the antifungal protein \textit{paf} gene from \textit{P. chrysogenum} (Sonderegger et al., 2016), and the CDS for the antifungal protein \textit{afpB} gene from \textit{P. digitatum} (Garrigues et al., 2016). All these DNA parts were cloned into pUPD2 by restriction-ligation and the resulting positive clones were confirmed by DNA sequencing. Importantly, once DNA parts are confirmed by sequencing and functionally validated (see sections below) there is no need for further sequencing or validation each time the DNA part is exchanged and used under the GB standards. Assemblies of these
minimal parts into more and more complex structures are shown in Table 1 and described in the sections below.

3.3. Transformation of *P. digitatum* with positive selection markers in GB transcriptional units

As a first step to validate the GB technology in fungi and demonstrate that the core plasmid vectors and even selected DNA parts of GB are interchangeable and fully usable in fungi, we set up an experiment to test fungal genetic transformation with the GB vectors, using both domesticated fungal-specific parts described above (i.e., the promoter FB001 and the terminator FB002) as well as parts taken directly from the plant GB library (for instance, the hygromycin resistance marker GB0211) (Fig. 3). Single TUs were assembled into pDGB3α2 as illustrated (Fig. 3A and 3D) for HygR (FB003) and G418R (FB009) (Table 1). The resulting constructs FB003 and FB009 were transformed into *P. digitatum* by ATMT. Positive *P. digitatum* colonies were recovered, monosporic resistant strains were isolated from them, genomic DNAs were purified and the genetic transformation was demonstrated by PCR amplification, following common procedures (data not shown). Most importantly, the positive results proved that compatible genetic elements domesticated for plants and the GB destination vectors used for transformation can be applied in fungi.

It was also very significant the demonstration that the resistance markers encoded in FB003 and FB009 confer improved resistance in *P. digitatum* compared to previously used plasmids. We had previously used the pBHt2 vector for HygR (Mullins et al., 2001) that has demonstrated effectiveness in different fungi in a number of previous studies. This vector contains the same
and hph elements although with minor single nucleotide changes compared to FB003, but does not contain a terminator. Three independent transformants generated by FB003 (Fig. 3B and C, in blue) showed enhanced levels of hygromycin resistance as compared to pBht2 transformants (in red). This FB003 is the very same part that will be further recycled and used in the subsequent constructions described in this study, following the GB procedures (see below).

A similar result occurred for FB009 conferring G418R encoded by nptII (Fig. 3E and Table 1). We had previously constructed a vector based on the kanMX gene (used for kanamycin/G418 resistance by the EUROSCARF yeast null mutant collection) under the regulation of the promoter P_{trpC} from A. nidulans (plasmid pCkan, unpublished results). However, this vector was a failure and its use was not practical due to the very low level of resistance obtained and the negative impact on fungal growth of the expression of the kanMX gene (Fig. 3E, strain PDJM001). Again, side-by-side comparisons demonstrated that strains transformed with FB009 (for instance, PDMG093 in Fig. 3E) showed a markedly improved resistance as compared with the previous plasmid pCkan. These experiments validate the use of FB003 and FB009 as standardized TUs for conferring positive selection in fungal transformation.

3.4. Assembly and transformation with multiple transcriptional units: Fluorescent tagging of P. digitatum

Binary assembly combines previously assembled TU constructs two by two, shifting from pDGB3α to pDGB3Ω vectors and vice versa. Through this method, the FB003 construct containing the positive selection marker Hyg^R
(Fig. 3A) was assembled with a TU producing the fluorescent protein YFP (FB026) into pDGB3Ω1R to produce FB027 (Fig. 4A). ATMT of \( P. \) digitatum with this construct efficiently generated YFP-tagged strains (PDMH021) that displayed intense cytosolic fluorescence uniformly distributed in both conidia and hyphal cells (Fig. 4B, C). Notably, the part GB0053 encoding YFP was recycled and successfully used in the fungus with no modifications of the plasmid previously used in plants (Sarrion-Perdigones et al., 2013). This suggests that other compatible GB parts (for instance N-terminal or C-terminal tags, NTAG or CTAG in Fig. 2) designed for the terminal tagging of proteins with fluorescent protein tags or detection/purification epitopes will be likewise useful to specifically label fungal cell structures or proteins.

3.5. A combinatorial experiment to demonstrate that the \( P. \) chrysogenum paf promoter results in antifungal protein production.

We are interested in the characterization and exploitation of the so-called antifungal proteins (AFPs). AFPs are small, cationic, cysteine-rich proteins that are usually secreted in large amounts by filamentous ascomycetes, and are specifically active against other fungi at micromolar concentrations, which make them great alternatives for the development of novel antifungal compounds. One of the best studied AFPs is the PAF protein from \( P. \) chrysogenum that is produced and secreted in high amounts by the producing fungus (Batta et al., 2009). \( P. \) digitatum encodes only one AFP which was named AfpB (Garrigues et al., 2016). AfpB could not be detected either in the wild-type strain of \( P. \) digitatum or in constitutive expression strains under the highly active \( PgpdA \) promoter that produced up to 1,000 times more \( afpB \) mRNA than the wild-type strain (Garrigues et al., 2016). Unexpectedly, these constitutive expression
strains showed a strong negative impact on fungal growth. Recently, we have shown that the exchange of the PAF coding sequence for the AfpB one in the paf gene from *P. chrysogenum*, and the subsequent transformation of this gene construct into *P. digitatum* led to the production of AfpB, which enabled its characterization as a highly active antifungal protein (Garrigues et al., 2017). However, it remained to be identified which regulatory element of the paf gene was responsible for the AfpB production, which was addressed in this work with the aid of the GB/FB technology.

The GB binary assembly was used to test the production of the *P. digitatum* antifungal protein AfpB under the regulation of either the Ppaf (FB033) or PgpdA (FB035) promoters, leaving constant the AfpB coding sequence and the Tpaf terminator (Fig. 5A). It must be mentioned that in this study we used a shorter intronless version of PgpdA (see above) to complement the previous work in which the long version of the promoter was unable to generate sufficient amounts of protein to allow for detection (Garrigues et al., 2016). Transformants harboring each construct were confirmed by PCR (Fig. 5B). Strains carrying the AfpB construction under the regulation of Ppaf showed a moderate reduction of growth (Fig. 5C, blue), but a high level of AfpB production (Fig. 5D, blue). On the contrary, strains carrying AfpB construction under the regulation of the constitutive intronless PgpdA showed a drastic reduction of growth (Fig. 5C, red) and were unable to produce AfpB (Fig. 5D, red). These results are consistent with our previous studies and confirm our conclusions about the negative effect on growth of the PgpdA promoter and its inefficiency to produce AfpB in *P. digitatum*, despite the high expression of the *afpB* gene (Garrigues et al., 2016). Moreover, neither of the two versions of the PgpdA used in this
(FB007 part) and the previous (Garrigues et al., 2016) study resulted in AfpB protein production. Previous reports demonstrated that the paf promoter showed greater efficiency than the A. nidulans gpdA constitutive promoter for the expression of reporter genes in P. chrysogenum (Polli et al., 2016). In this work, we further demonstrate with the aid of the GB combinatorial cloning strategy that the promoter of the P. chrysogenum paf gene is the main determinant for the high level of AfpB production in P. digitatum.

3.6. An example of fungal specific GB syntaxes for gene deletion.

We adapted the GB syntax to design a new modular cloning strategy for gene replacement through homologous recombination and dual selection (Fig. 6A). New amplicons were cloned into pUPD2 with suitable barcodes as TUs to be used as positive (hph, FB012 plasmid with AATG/GCTT barcodes) and negative (HSVtk, FB013 plasmid with GGAG/TACT barcodes) selection markers (see Table 1 for details). The rationale is to assemble these two universal modules with two specific DNA flanking fragments to target any genomic locus in a single restriction-ligation reaction. As proof-of-concept, we chose to target the hog1 gene (PDIG_79560) from P. digitatum. We selected the mitogen-activated protein kinase (MAPK) Hog1 because of its previously characterized role in fungal biology and pathogenesis (Wang et al., 2014). The FB014 plasmid (Table 1) contained a 1 Kb DNA fragment upstream of the hog1 coding sequence amplified from genomic DNA with primers OJM539 and OJM540 (Table 2), while FB015 contained a 1 Kb DNA fragment downstream of the hog1 amplified with primers OJM541 and OJM542. The four pUPD2 constructs were assembled in the order FB013, FB014, FB012 and FB015 based on the design of the barcodes into the pDGB3α2 binary vector to obtain
FB022. Sets of newly designed universal primers were used to verify correct vector assembly by PCR amplification (Fig. 6B and 6C). This strategy to obtain gene replacement constructions drastically reduces the time required to generate and confirm deletion vectors and allows for the recycling of selection markers from the GB library without sequencing every time a new assembly is constructed.

The resulting binary vector FB022 was directly used to knock out the hog1 gene following the ATMT protocol adapted in our laboratory (Harries et al., 2015) (Fig. 6D). Three independent transformants (in red) were confirmed by PCR amplification of genomic DNA using outside primers and specific primers from the positive selection marker to discriminate positive null mutants from negative ectopic insertions or wild-type genotypes (Fig. 6E). Two of these positive Δhog1 strains shown in Fig. 6E were selected for further analyses to confirm the phenotype of the null mutants. Transformants grown on PDA plates showed no major differences in size of mycelial growth compared to the parental strain CECT 20796 (Fig. 6F). Nevertheless, growth of null mutants in the presence of sorbitol or NaCl, which produce hyperosmotic stress, was reduced as previously described in P. digitatum (Wang et al., 2014) and in other Δhog1 mutants of Fusarium oxysporum (Segorbe et al., 2016), Verticillium dahliae (Wang et al., 2016) or Fusarium graminearum (Ramamoorthy et al., 2007) confirming the identity of the Δhog1 null mutants (Fig. 6G). In summary, this newly described procedure based on the GB modular cloning and grammar allows the highly efficient and quick construction of plasmids that can be used to knock out fungal genes with ready-to-use standardized and universal genetic elements.
4. Concluding remarks and future developments

By taking advantage of the capability of *A. tumefaciens* to transform both plants and filamentous fungi, we have successfully adapted the GB technology for the ATMT of fungi. We propose the name of FungalBraid (FB) for this new branch of GB and open the approach and resources for the contributive use by the fungal research community. The *hph* and *nptII* markers, promoters and terminators domesticated in this work are undoubtedly amongst the most frequently used in fungal transformation (Fang et al., 2006) and cover our current needs for *P. digitatum*. We anticipate that the future use of the FB system by the community following the Phytobrick standard will exponentially expand the available parts and resources with additional markers, promoters and so on. Domesticated DNA parts can be easily designed using the tools provided on the GB web page, and produced through PCR mutagenesis or by synthetic gene design. The basic plasmid toolbox described in this report covering resistance markers (i.e., FB003 or FB009) or fluorescent tagging (FB027) constructs can be directly used, in any case, in many basic fungal transformation studies. In addition, the system enables reusability of genetic parts and direct comparison and reproducibility of experiments among different laboratories or fungal species. Finally, it is expected that the repertoire of more sophisticated and fungal-compatible GB tools already developed for plants, such as switchable exogenous transcriptional regulators, genetic circuits or CRISPR/Cas9 parts (Vazquez-Vilar et al., 2016; Vazquez-Vilar et al., 2017), will be interchangeable and usable in fungi. This technology will hopefully contribute to speed up the development of fungal synthetic biology.
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Figure Legends

Fig. 1. The GoldenBraid (GB) 3.0 cloning and DNA assembly strategy.
Outline of the three GB cloning and modular assembly steps and their connections: Domestication, Multipartite Assembly and Binary Assembly, which are dotted-line boxed. Flanking type IIS restriction sites and their cuts are indicated in orange \((Bm, BsmBI)\) or red \((Ba, Bsai)\) boxes with the direction of their cuts as arrows. The four-nucleotide barcodes are shaded in grey and exemplified A, B, C or D to illustrate the way (order and position) they facilitate the predefined base-pair assembly of DNA parts. These barcodes are indicated in the domestication and multipartite assembly steps, but omitted in the binary assembly for simplicity. Different DNA parts are color and pattern coded. Nucleotide level details of the DNA parts and plasmids are shown in Supplementary Figs. S1 and S2. The code of color shading is maintained in the Supplementary Figures. Additional barcodes in the alpha and omega destination plasmids allow for the correct assembly and order of the “endless braid” binary assembly (see Supplemental Figure S2). The three different antibiotic resistance genes chloramphenicol (Cam), kanamycin (Kan) and spectinomycin (Spm) are also indicated in the different plasmids. Further details are explained in the text. Adapted from (Sarrion-Perdigones et al., 2013; Vazquez-Vilar et al., 2017).

Fig. 2. The GB3.0 grammar and structures used in this work. Schematic overview of a Transcriptional Unit (TU) where 10 standard GB classes (from
DIST to TERM) and the 4-nucleotide DNA barcodes are depicted. Adapted from (Sarrion-Perdigones et al., 2013; Patron et al., 2015).

Fig. 3. Genetic transformation of *P. digitatum* with GB resistance markers.

Results shown demonstrate the transformation with hygromycin (A, B and C) or geneticin (D and E) resistance markers. (A) and (D), schematic view of the binary plasmids used for transformation (FB003 and FB009) and their constituent modular DNA parts (FB001, FB002, FB005 and GB0211). Radial growth (B), and images (C) of the parental strain (CECT 20796, black), transformants obtained with the FB003 construct (PDMH311, PDMH321 and PDMH351, in blue) and transformants obtained with the previous pBHt2 plasmid (PDMH211, PDMH221 and PDMH231, in red), grown on PDA plates or plates supplemented with hygromycin as indicated. Images (E) of the parental strain (CECT 20796, black), one transformant obtained with the FB009 construct (PDMG093, in orange) and one transformant obtained with the unpublished pCkanMx plasmid (PDJM001, in purple), grown on PDA plates or on plates supplemented with G418 as indicated.

Fig. 4. Generation of fluorescently tagged *P. digitatum* strains. (A)

Schematic diagram of the binary assembly of vector FB027 containing the hygromycin resistance module FB003 and the FB026 module that expresses the yellow fluorescent protein (YFP) from the GB library under a fungal-specific promoter and terminator. Bright field and fluorescent images of conidia (B) and mycelium (C), from the parental strain CECT 20796, and one representative transformant obtained with the FB027 construct (PDMH021).
Fig. 5. Generation of *P. digitatum* transformant strains for AfpB production by a combinatorial experiment using FungalBraid. (A) Schematic diagram of the binary assembly of vectors FB033 and FB035 with FB003 to obtain the final binary vectors, FB036 and FB037. The different promoters compared in this study are P*paf* from *P. chrysogenum* (dark blue) and the intronless *Pgpd*A from *A. nidulans* (red). (B) PCR amplification of *P. digitatum* genomic DNA to show positive *afpB* transformants carrying either the *P*paf* (dark blue) or *Pgpd*A (red) promoters. The primer pairs used to distinguish between transformants are indicated in the figure. (C) Growth of the CECT 20796 parental strain (black) and three independent positive *afpB* transformants carrying *P*paf* (dark blue) or *Pgpd*A (red) on PDA plates after 5 days of incubation at 25 °C. (D) Analysis by SDS-PAGE and Coomassie blue staining of proteins present in the supernatants of AfpB transformant strains carrying the *P*paf* (dark blue) or *Pgpd*A (red) promoters. Two µg of pure AfpB and the supernatant of parental strain CECT 20796 were added as controls.

Fig. 6. Example of fungal specific GB syntaxes. Construct design and genetic transformation for *hog1* gene disruption by homologous recombination and dual selection. (A) Diagram of the GB grammar used to generate gene disruption constructs. Plasmids FB012 and FB013 are universal positive (*hph* for *Hyg*R) and negative (*HSVtk* for *F2dU*S) selection markers with AATG/GCTT and GGAG/TACT DNA barcodes, respectively, designed to be assembled in a single multipartite assembly with 5’ and 3’ flanking regions of the gene of interest. In this experiment, 5’ and 3’ flanking regions are contained
in plasmids FB014 and FB015, respectively. (B) Schematic representation of
the multipartite assembly (FB022 in the example of the *P. digitatum* hog1 gene,
see text for further details) and the different universal primers used for PCR
amplification to verify correct assembly. (C) PCR verification of three
independent clones (clone 2, 8, and 10) with the three different primer
combinations (a, b, and c) that produce amplicons indicating the correct
orientation of modular pieces. (D) Diagrams of the gene replacement. The hog1
gene in the genomic DNA (gDNA) of the parental strain CECT 20796 (top); the
FB022 vector designed for gene disruption (middle); and the Δhog1 disrupted
locus (bottom) are shown. All primers used for PCR analysis of transformants
are indicated in the figure. (E) PCR verification of genomic DNA to show
positive Δhog1 transformants (in red) of *P. digitatum*, with different primer pairs
as indicated. Outside primers 558/559 produced fragment sizes of 2.9 Kb or 3.8
kb in length from CECT 20796 and ectopic transformants (PDMG5131 from this
transformation experiment and PDMG612 from a previous transformation
experiment) or from positive homologous recombination transformants
(PDMG5121, PDMG5135 and PDMG5187), respectively. Primers 197/232
revealed the presence of the hygromycin resistant cassette in all transformants.
Additional controls included are the plasmid control (FB022) and the negative
control (ddH2O). (F) Growth of the parental CECT 20796 and disruption strain
PDMG5121 (in red) on PDA plates after 6 days of incubation at 25 ºC. (G)
Growth on 24-well plates of 10-fold serial dilutions of conidia (indicated at the
top) of different *P. digitatum* strains (Δhog1 strains in red; parental and ectopic
strains in black) in PDA, PDA supplemented with sorbitol and PDA
supplemented with NaCl at the concentrations indicated in the figure.


Figure 1 (Hernanz-Koers et al., 2018)
Figure 2 (Hernanz-Koers et al., 2018)
Figure 3 (Hernanz-Koers et al., 2018)

A

FB001 P trpC

GB0211 hph

FB002 T tub

FB003

C

CECT 20796 PDMH231 PDMH311

PDA

PDA 25 µg/mL Hyg

PDA 75 µg/mL Hyg

D

FB001

FB005 nptII

FB002 T tub

FB009

E

CECT 20796 PDMG093 PDJM001

PDA

PDA 25 µg/mL G418
Figure 4 (Hernanz-Koers et al., 2018)

A

FB026

FB008

FB003

FB002

GB0053

FB001

GB0211

FB007

FB027

T

YFP

P

P

hph

T


B

CECT 20796

PDMH021


C

CECT 20796

PDMH021
Figure 5 (Hernanz-Koers et al., 2018)

A

\[ \text{afpB} \quad \text{Ppaf} \quad \text{FB033} \]

+ \[ \text{afpB} \quad \text{PpgdA} \quad \text{FB035} \]

\[ \text{afpB} \quad \text{hph} \quad \text{FB036/FB037} \]

B

\[
\begin{array}{c}
\text{2.0 kb} \\
\text{1.6 kb} \\
\text{[483-484]} \\
\text{[509-484]}
\end{array}
\]

C

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<tr>
<th>CECT 20796</th>
<th>PDSG400</th>
<th>PDSG420</th>
<th>PDSG438</th>
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<tbody>
<tr>
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D

6.5 kDa →
Figure 6 (Hernanz_Koers et al., 2017)

A

<table>
<thead>
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<td>TGAC</td>
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<tr>
<td>5' FLANK</td>
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<td>3' FLANK</td>
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</tbody>
</table>

FB013  FB014  FB012  FB015

B

HSVtk  5'  hph  3'

FB022  [pDGB3α2]

533  311  312  232  197  534

C

1.3 kb  3.2 kb  2.7 kb

a  b  c

[533-311]  [312-232]  [197-534]

D

500 pb

HSVtk  5'  hph  3'

FB022  gDNA (CECT 20796)  gDNA (Δhog1)

533  311  558  197  232  534  559

E

CECT 20796  PDMG5121 (Δhog1)

3.8 Kb  2.9 Kb  0.8 Kb

[558-559]  [197-232]

F

G

CECT 20796  PDMG5121  PDMG5135  PDMG5131

Control  1.2 M Sorbitol  0.5 M NaCl
Supplemental Figure S1 (Hernanz-Koers et al., 2018)

**Diagram Description:**

- **pUPD2** contains a region of DNA labeled with BsmBI restriction sites.
- **LacZ** is adjacent to pUPD2, with BsmBI and Bsal restriction sites.
- A CamR marker is present, suggesting a selectable marker for bacterial growth.
- T4 Ligation step results in the combination of DNA parts.
- Bsal restriction step follows the ligation, further modifying the DNA sequence.

**DNA Sequences:**

- pUPD2 sequence:
  
  AGTGGTCTCGTGAGACCGCTACCAGAGCCTCTGC
  
- LacZ sequence:
  
  CGCCGTCTCGCTCG1234GAGCGAGACGGCGGCGGCAGAGCGAGC1234
  
  BsmBI restriction sites are indicated at specific positions.

**Restriction Enzymes:**

- **BsmBI** cuts at specific bases.
- **Bsal** (BamHI) cuts at specific bases.

**Markers:**

- **CamR** indicates a selectable marker for bacterial growth.
- **F** and **R** denote the 5' and 3' ends of the DNA sequences.

**Figure Key:**

- The diagram uses purple and orange arrows to denote the direction of DNA movement and restriction enzyme cuts.
- Key positions 1234 and 5678 indicate specific nucleotide locations within the DNA sequences.
Supplemental Figure S1. Domestication of GB DNA parts under the v3.0 standard.

This Supplemental Figure is an enlargement of the “Domestication” step in Figure 1 of the main text. DNA parts (purple) are domesticated either by amplifying the desired sequence with standard GB primers (F and R) or by gene synthesis. GB primers include approximately 20 nucleotides of gene-specific sequence (purple) and a tail region that includes a BsmBI recognition site (orange) for cloning into pUPD2, and the four-nucleotide barcode (“1234” and “5678”, in grey). The DNA part is cloned into pUPD2 in a one pot (dotted line) simultaneous restriction-ligation reaction that uses BsmBI as the restriction enzyme and T4 DNA ligase. The resulting GB/FB part (FB0x, in this example) is selected under Cam resistance and white/blue color (LacZ selection), purified and stored as a reusable DNA part. FB0x is cleavable by Bsal to produce a dsDNA with “1234” and “5678” flanking overhangs, which will be used in the subsequent “Multipartite Assembly” step (Figure 1). BsmBI and Bsal recognition sequences are depicted in orange and red, respectively, in the DNA sequence. Outside the Bsal sites, plasmid pUPD2 also contains BtgZI restriction sites (not depicted). Modified from Figure 3 of Sarrion-Perdigones et al. (Sarrion-Perdigones, A., Vazquez-Vilar, M., Palací, J., Castelijns, B., Forment, J., Ziarso, P., et al., 2013, GoldenBraid 2.0: A comprehensive DNA assembly framework for plant synthetic biology. Plant Physiol., 162, 1618-163). Additional information and further details can be found at the GoldenBraid web page (https://gbcloning.upv.es/).
Supplemental Figure S2 (Hernanz-Koers et al., 2018)

**α1**

\[\text{EcoRI} \quad \text{BsmBI} \quad \text{Bsal} \quad \text{Bsal} \quad \text{BsmBI} \quad \text{EcoRI}\]

\[\text{GAATTCGTCTCAGGAGAGACC} \quad \text{GGTCTCTCGCTTGAGACGAAGCTT} \]
\[\text{CTTAAGCAGAGTCCTCTCTGTG} \quad \text{CCAGAGCGAGTGACTCTGGTTAAG}\]

**α2**

\[\text{HindIII} \quad \text{BsmBI} \quad \text{Bsal} \quad \text{Bsal} \quad \text{BsmBI} \quad \text{HindIII}\]

\[\text{AAGCTTGTCTCAGGAGAGACC} \quad \text{GGTCTCTCGTTGAGACGAAGCTT} \]
\[\text{TTCGAAGCAGAGTCCTCTCTGTG} \quad \text{CCAGAGCGAGTGACTCTGGTTAAG}\]

**α1R**

\[\text{BamHI} \quad \text{BsmBI} \quad \text{Bsal} \quad \text{Bsal} \quad \text{BsmBI} \quad \text{BamHI}\]

\[\text{GGATCCGTCTCAGGAGAGCGACG} \quad \text{GGTCTCTCGCTGTCATGAGACGATATC} \]
\[\text{CCTAGGCCAGAGTCCTCTCGTG} \quad \text{CCAGAGTGCGACTCTGCTATAG}\]

**α2R**

\[\text{EcoRV} \quad \text{BsmBI} \quad \text{Bsal} \quad \text{Bsal} \quad \text{BsmBI} \quad \text{EcoRV}\]

\[\text{GATATCGTCTCAGTGAGCGACG} \quad \text{GGTCTCTCGCTGTCATGAGACGATATC} \]
\[\text{CTATAGCAGAGTCACTCTCTTG} \quad \text{CCAGAGTGCGACTCTGCTATAG}\]

**Ω1**

\[\text{BamHI} \quad \text{Bsal} \quad \text{BsmBI} \quad \text{BsmBI} \quad \text{Bsal} \quad \text{BamHI}\]

\[\text{GGATCCGTTCTAGGAGAGCG} \quad \text{GGTCTCTCGCTGTCATGAGACGATATC} \]
\[\text{CCTAGCCAGAGTCTCTCTTG} \quad \text{CCAGAGTGCGACTCTGCTATAG}\]

**Ω2**

\[\text{EcoRV} \quad \text{Bsal} \quad \text{BsmBI} \quad \text{BsmBI} \quad \text{Bsal} \quad \text{EcoRV}\]

\[\text{GATATCGTCTCAGTGAGCGACG} \quad \text{GGTCTCTCGCTGTCATGAGACGATATC} \]
\[\text{CTATAGCAGAGTCACTCTCTTG} \quad \text{CCAGAGTGCGACTCTGCTATAG}\]

**Ω1R**

\[\text{EcoRI} \quad \text{Bsal} \quad \text{BsmBI} \quad \text{BsmBI} \quad \text{Bsal} \quad \text{EcoRI}\]

\[\text{GAATTCGTCTCAGGAGAGCGACG} \quad \text{GGTCTCTCGCTGTCATGAGACGATATC} \]
\[\text{CCTAGCCAGAGTCTCTCTTG} \quad \text{CCAGAGTGCGACTCTGCTATAG}\]

**Ω2R**

\[\text{HindIII} \quad \text{Bsal} \quad \text{BsmBI} \quad \text{BsmBI} \quad \text{Bsal} \quad \text{HindIII}\]

\[\text{AAGCTTGTCTCAGGAGAGCGACG} \quad \text{GGTCTCTCGCTGTCATGAGACGATATC} \]
\[\text{TTCGAAGCAGAGTCACTCTCTTG} \quad \text{CCAGAGTGCGACTCTGCTATAG}\]
**Supplemental Figure S2. Cloning cassettes of GoldenBraid v3.0 destination vectors.**

There are eight destination vectors in GB v3.0 (pDGB3): four for each of the alpha (α) or omega (Ω) subsets, coded as 1, 1R, 2 and 2R. DNA parts can be excised from the pUPD2 backbone by *Bsa*I digestion (Supplemental Figure S1) and assembled in order (as determined by the barcodes shown in Figure 2) into any of the four alpha destination plasmids in the first “multipartite assembly” (Figure 1). From here on, an endless and customized number of “binary assembly” steps (Figure 1) can be designed that allow the assembly—each time—of two alpha (or omega) vectors into a single omega (or alpha) vector, alternatively, with the use of *Bsm*BI (or *Bsa*I) enzymes, producing a continuous intertwined “braid” of gene constructs. The four different options (1, 1R, 2 and 2R) of each alpha/omega vector permit the desired order (1 precedes 2) and orientation (direct or reverse) of TUs. There are three additional four-nucleotide barcodes incorporated in the eight destination vectors (color-coded as light green, red and blue) that result from *Bsm*BI/*Bsa*I restriction and determine the ordered binary assembly. Grey color shading in pDGB3α2R and pDGBΩ2R vectors refers to a nucleotide shared by two adjacent nucleotide barcodes. pDGB3 plasmids also incorporate distinctive restriction sites flanking the cloning cassette (*Eco*RI, *Hind*III, *Bam*HI or *Eco*RV) as watermarks for plasmid identification. *Bsa*I cleavage sequences are marked in red, and *Bsm*BI cleavage sequences in orange. Modified from Figure 4 of Sarrion-Perdigones et al. (Sarrion-Perdigones, A., Vazquez-Vilar, M., Palací, J., Castelijns, B., Forment, J., Ziarsolo, P., et al., 2013, GoldenBraid 2.0: A comprehensive DNA assembly framework for plant synthetic biology. Plant Physiol., 162, 1618-163). Additional information and further details can be found at the GoldenBraid web page (https://gbcloning.upv.es/).