Hernanz-Koers, M.; Gandía-Gómez, M.; Garrigues-Cubells, SM.; Manzanares-Mir, PM.; Yenush, L.; Orzáez Calatayud, DV.; Marcos -Lopez, JF. (07-2). FungalBraid: A GoldenBraid-based modular cloning platform for the assembly and exchange of DNA elements tailored to fungal synthetic biology. Fungal Genetics and Biology. 116:51-61. https://doi.org/10.1016/j.fgb.2018.04.010

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

Miguel Hernanz-Koers [a §], Mónica Gandía [a §], Sandra Garrigues [a], Paloma Manzanares [a], Lynne Yenush [b], Diego Orzaez [b], and Jose F. Marcos [a *]

[a] Department of Food Biotechnology, Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC), Avda Agustín Escardino 7, 46980 Paterna, Valencia, Spain.

[b] Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universitat Politècnica de València-Consejo Superior de Investigaciones Científicas (CSIC) 46022 Valencia, Spain.

[§] These authors contributed equally to this work and shall be considered first co-authors.

[*] Corresponding author: Dr. Jose F. Marcos. Instituto de Agroquímica y Tecnología de Alimentos (IATA) - CSIC, Avenida Agustín Escardino 7, Paterna 46980, Valencia, Spain. e-mail: jmarcos@iata.csic.es. Phone: (34)963.900.022. Fax: (34)963.636.301

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 Hyg^R 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

103 suitable, and thus cannot be extended to other fungi. Nowadays, it is well
104 known that ATMT enables the gene disruption and genetic transformation of an
105 increasingly wide number of fungal species by using virtually the same protocol,
106 including non-model fungi for which molecular genetic tools are not well
107 developed (de Groot et al., 1998; Michielse et al., 2005; Frandsen, 2011).
108 Moreover, the binary plasmids and *Agrobacterium* strains used for ATMT of
109 fungi are fairly standard, and common to those used in plant transformation. In
110 this study, we take advantage of these facts to implement and demonstrate the
111 feasibility of the GB and PhytoBrick standards for the ATMT of filamentous
112 fungi, and the exchangeability of the system between plants and fungi.
113 *Penicillium digitatum* is a specific postharvest pathogen of citrus fruit that
114 causes the green mold citrus decay, producing very important economic losses
115 in one of the main fruit tree crops worldwide (Palou, 2014). The main method to
116 control this and other postharvest pathogens is the massive use of chemical
117 fungicides, which causes the appearance of resistance (Holmes and Eckert,
118 1999) and puts human health and the environment at risk. Molecular and
119 genetic studies have been recently initiated to characterize the *P. digitatum*
120 biology and search for novel control strategies. These include the sequencing of
121 the genome of different *P. digitatum* strains (Marcet-Houben et al., 2012; Sun et
122 al., 2013), which has accelerated the use of reverse genetic approaches to
123 study the biological role and function of specific genes related to virulence and
124 pathogenesis (Zhang et al., 2013a; Zhang et al., 2013b; Gandía et al., 2014;
125 Wang et al., 2014; Harries et al., 2015; Gandía et al., 2016; Garrigues et al.,
126 2016; Ma et al., 2016; de Ramón-Carbonell and Sánchez-Torres, 2017). We
127 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 ºC. 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 ºC depending on the vector. The *A. tumefaciens* AGL-1 strain was grown in LB medium supplemented with 20 µg/mL rifampicin at 28 ºC.

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

177 fluorescent protein (YFP) marker using the hygromycin resistance gene as the
178 fungal selection marker.
179 In order to generate specific gene constructs to compare the efficiency of
180 P*paf* and P*gpdA* promoters in the production and secretion of the antifungal
181 protein AfpB from *P. digitatum*, two different multipartite assemblies
182 (FB036/FB037) were generated. These contained the TU for the expression of
183 the *afpB* coding sequence (FB031) and the T*paf* terminator (FB030), under the
184 control of either the P*paf* (FB029) or P*gpdA* (FB007) promoters. Plasmids
185 FB029, FB030, FB031 were obtained by direct cloning of synthetic gene
186 fragments into pUPD2, which allowed gene domestication in a simple and
187 inexpensive way.
188 To generate the specific construct to disrupt the *P. digitatum hog1* gene by
189 homologous recombination and dual selection, multipartite assembly of four
190 single modular units (FB012, FB013, FB014 and FB015) was performed to
191 obtain the binary vector FB022 in a pDGB3α2 backbone. FB012 was obtained
192 using FB003 as a template to amplify the TU encoding for Hyg^R with OJM535
193 and OJM536 primers with specific barcodes, and used as the positive selection
194 marker. FB013 was obtained from FB006 with OJM537 and OJM538 primers
195 adapting the GB grammar to allow its reverse orientation (Table 1) and used as
196 negative selection marker. FB014 and FB015 contained two 1 Kb DNA
197 fragments upstream and downstream of the *hog1* coding sequence, and
198 obtained with primers OJM539 and OJM540 (FB014) or with OJM541 and
199 OM542 (FB015).
200 Positive clones and correct assembly were confirmed by restriction
201 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 10^4 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 10^6 conidia/mL suspensions of

226 different strains were used. The YFP fluorescence was visualized in a Nikon
227 E90i fluorescence microscope with excitation/emission wavelengths of 488/535
228 nm, respectively. The images were captured with a 40X objective using the
229 software NIS-Elements BR v2.3 (Nikon).

230 *2.5. Determination of AfpB production*

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

239 *2.6. Sensitivity of null Δhog1 mutants*

240 To confirm the identity of the Δ*hog1* null strains, their sensitivity to different
241 compounds was tested. Growth in PDA medium supplemented with 1.2 M
242 sorbitol as osmotic stabilizer or 0.5 M NaCl was observed after incubating the
243 strains on 24-well plates at 25 ºC for 4 days. Five µL of serial 10-fold dilutions of
244 conidia (from 1 x 10^5 to 1 x 10^3 conidia/mL) were added to each well.
245

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 *Bsm*BI, *Bsa*I and *Btg*ZI, and to incorporate flanking four-nucleotide barcodes and *Bsm*BI 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 *Bsm*BI 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 *Bsa*I 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 (*Bsa*I) 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 *Bsa*I 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.,

296 Addgene, https://www.addgene.org/). Genetic elements amplified and
297 domesticated by the PCR approach with the aid of specifically designed primers
298 (see Table 2) were: the P*trpC* promoter from *A*. *nidulans* (Hamer and
299 Timberlake, 1987) amplified from pBHt2 (Mullins et al., 2001); a short intronless
300 version of the P*gpdA* promoter (Punt et al., 1990) and the T*trpC* terminator
301 (Hamer and Timberlake, 1987) from *A*. *nidulans*, both amplified from
302 pBARGPE1 (Pall and Brunelli, 1993); the T*tub* terminator from *N. crassa*
303 amplified from pGKO2 (Khang et al., 2005); the positive selection marker *nptII*
304 for kanamycin/G418 resistance from *E. coli* (Mazodier et al., 1985) amplified
305 from GB0034 (https://gbcloning.upv.es/); and the complete TU of the negative
306 marker HSV*tk* for F2dU^{S} that includes the P*gpdA* promoter from
307 *Cochliobolus heterostrophus*, the CDS for thiamine kinase (*tk*) from Herpes
308 simplex virus (HSV), and the T*tub* terminator from *N*. *crassa* amplified from
309 pGKO2 (Khang et al., 2005). Single band amplicons were confirmed by gel
310 electrophoresis before cloning into pUPD2.
311 Three additional genetic elements were domesticated as synthetic DNAs
312 (Table 3): the P*paf* promoter and T*paf* terminator from the antifungal protein *paf*
313 gene from *P*. *chrysogenum* (Sonderegger et al., 2016), and the CDS for the
314 antifungal protein *afpB* gene from *P. digitatum* (Garrigues et al., 2016).
315 All these DNA parts were cloned into pUPD2 by restriction-ligation and the
316 resulting positive clones were confirmed by DNA sequencing. Importantly, once
317 DNA parts are confirmed by sequencing and functionally validated (see sections
318 below) there is no need for further sequencing or validation each time the DNA
319 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 Hyg^R (FB003) and $G418^R$ (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 Hyg^R (Mullins et al., 2001) that has demonstrated effectiveness in different fungi in a number of previous studies. This vector contains the same

P*trpC* 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 $G418^R$ 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 P*gpdA* promoter that produced up to 1,000 times more *afpB* mRNA than the wild-type strain (Garrigues et al., 2016). Unexpectedly, these constitutive expression

395 strains showed a strong negative impact on fungal growth. Recently, we have
396 shown that the exchange of the PAF coding sequence for the AfpB one in the
397 *paf* gene from *P*. *chrysogenum*, and the subsequent transformation of this gene
398 construct into *P. digitatum* led to the production of AfpB, which enabled its
399 characterization as a highly active antifungal protein (Garrigues et al., 2017) .
400 However, it remained to be identified which regulatory element of the *paf* gene
401 was responsible for the AfpB production, which was addressed in this work with
402 the aid of the GB/FB technology.
403 The GB binary assembly was used to test the production of the *P.*
404 *digitatum* antifungal protein AfpB under the regulation of either the P*paf* (FB033)
405 or P*gpdA* (FB035) promoters, leaving constant the AfpB coding sequence and
406 the T*paf* terminator (Fig. 5A). It must be mentioned that in this study we used a
407 shorter intronless version of P*gpdA* (see above) to complement the previous
408 work in which the long version of the promoter was unable to generate sufficient
409 amounts of protein to allow for detection (Garrigues et al., 2016). Transformants
410 harboring each construct were confirmed by PCR (Fig. 5B). Strains carrying the
411 AfpB construction under the regulation of P*paf* showed a moderate reduction of
412 growth (Fig. 5C, blue), but a high level of AfpB production (Fig. 5D, blue). On
413 the contrary, strains carrying AfpB construction under the regulation of the
414 constitutive intronless P*gpdA* showed a drastic reduction of growth (Fig. 5C,
415 red) and were unable to produce AfpB (Fig. 5D, red). These results are
416 consistent with our previous studies and confirm our conclusions about the
417 negative effect on growth of the P*gpdA* promoter and its inefficiency to produce
418 AfpB in *P. digitatum*, despite the high expression of the *afpB* gene (Garrigues et
419 al., 2016). Moreover, neither of the two versions of the P*gpdA* 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 (HSV*tk*, 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

445 FB022. Sets of newly designed universal primers were used to verify correct
446 vector assembly by PCR amplification (Fig. 6B and 6C). This strategy to obtain
447 gene replacement constructions drastically reduces the time required to
448 generate and confirm deletion vectors and allows for the recycling of selection
449 markers from the GB library without sequencing every time a new assembly is
450 constructed.

451 The resulting binary vector FB022 was directly used to knock out the *hog1*
452 gene following the ATMT protocol adapted in our laboratory (Harries et al.,
453 2015) (Fig. 6D). Three independent transformants (in red) were confirmed by
454 PCR amplification of genomic DNA using outside primers and specific primers
455 from the positive selection marker to discriminate positive null mutants from
456 negative ectopic insertions or wild-type genotypes (Fig. 6E). Two of these
457 positive Δ*hog1* strains shown in Fig. 6E were selected for further analyses to
458 confirm the phenotype of the null mutants. Transformants grown on PDA plates
459 showed no major differences in size of mycelial growth compared to the
460 parental strain CECT 20796 (Fig. 6F). Nevertheless, growth of null mutants in
461 the presence of sorbitol or NaCl, which produce hyperosmotic stress, was
462 reduced as previously described in *P. digitatum* (Wang et al., 2014) and in other
463 Δ*hog1* mutants of *Fusarium oxysporum* (Segorbe et al., 2016), *Verticillium*
464 *dahliae* (Wang et al., 2016) or *Fusarium graminearum* (Ramamoorthy et al.,
465 2007) confirming the identity of the Δ*hog1* null mutants (Fig. 6G). In summary,
466 this newly described procedure based on the GB modular cloning and grammar
467 allows the highly efficient and quick construction of plasmids that can be used to
468 knock out fungal genes with ready-to-use standardized and universal genetic
469 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.

Acknowledgments

This work was funded by grants BIO2015-68790-C2-1-R and BIO2016-78601-R from the “Ministerio de Economía y Competitividad” (MINECO, Spain). SG was recipient of a predoctoral scholarship (FPU13/04584) within the FPU program from “Ministerio de Educación, Cultura y Deporte” (MECD, Spain). We acknowledge the excellent technical assistance of Tania Campos and the help in the microscopy experiments of José M. Coll-Marqués (IATA, Valencia, Spain). We also thank Dr. Pilar Moya (Universitat Politècnica de València, Spain) for helpful discussions during the initial stages of this project.

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*, *Bsm*BI) or red (*Ba*, *Bsa*I) 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 P*gpdA* 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 P*gpdA* (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 P*gpdA* (red) on PDA plates after 5 days of incubation at 25 ºC. (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 P*gpdA* (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 (HSV*tk* 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

578 in plasmids FB014 and FB015, respectively. (B) Schematic representation of
579 the multipartite assembly (FB022 in the example of the *P. digitatum hog1* gene,
580 see text for further details) and the different universal primers used for PCR
581 amplification to verify correct assembly. (C) PCR verification of three
582 independent clones (clone 2, 8, and 10) with the three different primer
583 combinations (a, b, and c) that produce amplicons indicating the correct
584 orientation of modular pieces. (D) Diagrams of the gene replacement. The *hog1*
585 gene in the genomic DNA (gDNA) of the parental strain CECT 20796 (top); the
586 FB022 vector designed for gene disruption (middle); and the Δ*hog1* disrupted
587 locus (bottom) are shown. All primers used for PCR analysis of transformants
588 are indicated in the figure. (E) PCR verification of genomic DNA to show
589 positive Δ*hog1* transformants (in red) of *P. digitatum*, with different primer pairs
590 as indicated. Outside primers 558/559 produced fragment sizes of 2.9 Kb or 3.8
591 kb in length from CECT 20796 and ectopic transformants (PDMG5131 from this
592 transformation experiment and PDMG612 from a previous transformation
593 experiment) or from positive homologous recombination transformants
594 (PDMG5121, PDMG5135 and PDMG5187), respectively. Primers 197/232
595 revealed the presence of the hygromycin resistant cassette in all transformants.
596 Additional controls included are the plasmid control (FB022) and the negative
597 control (ddH_2O). (F) Growth of the parental CECT 20796 and disruption strain
598 PDMG5121 (in red) on PDA plates after 6 days of incubation at 25 ºC. (G)
599 Growth on 24-well plates of 10-fold serial dilutions of conidia (indicated at the
600 top) of different *P. digitatum* strains (Δ*hog1* strains in red; parental and ectopic
601 strains in black) in PDA, PDA supplemented with sorbitol and PDA
602 supplemented with NaCl at the concentrations indicated in the figure.

References

Batta, G., Barna, T., Gáspári, Z., Sándor, S., Kövér, K.E., Binder, U., Sarg, B., Kaiserer, L., Chhillar, A.K., Eigentler, A., Leiter, E., Hegedüs, N., Pócsi, I., Lindner, H., Marx, F., 2009. Functional aspects of the solution structure and dynamics of PAF - A highly-stable antifungal protein from *Penicillium chrysogenum*. FEBS J. 276**,** 2875-2890.

Boecker, S., Zobel, S., Meyer, V., Süssmuth, R.D., 2016. Rational biosynthetic approaches for the production of new-to-nature compounds in fungi. Fungal Genet. Biol. 89**,** 89-101.

Brown, G.D., Denning, D.W., Gow, N.A.R., Levitz, S.M., Netea, M.G., White, T.C., 2012. Hidden killers: Human fungal infections. Sci. Transl. Med. 4**,** 165rv13.

de Groot, M.J.A., Bundock, P., Hooykaas, P.J.J., Beijersbergen, A.G.M., 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat. Biotech. 16**,** 839-842.

de Ramón-Carbonell, M., Sánchez-Torres, P., 2017. The transcription factor PdSte12 contributes to *Penicillium digitatum* virulence during citrus fruit infection. Postharvest Biol. Tec. 125**,** 129-139.

Engler, C., Gruetzner, R., Kandzia, R., Marillonnet, S., 2009. Golden Gate shuffling: A one-pot DNA shuffling method based on type IIs restriction enzymes. PLoS ONE 4**,** e5553.

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

Fang, W., Pei, Y., Bidochka, M.J., 2006. Transformation of *Metarhizium anisopliae* mediated by *Agrobacterium tumefaciens*. Can. J. Microbiol. 52**,** 623-626.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L., Gurr, S.J., 2012. Emerging fungal threats to animal, plant and ecosystem health. Nature 484**,** 186-94.

Frandsen, R.J., 2011. A guide to binary vectors and strategies for targeted genome modification in fungi using *Agrobacterium tumefaciens*-mediated transformation. J. Microbiol. Meth. 87**,** 247-62.

Gandía, M., Harries, E., Marcos, J.F., 2014. The myosin motor domain-containing chitin synthase PdChsVII is required for development, cell wall integrity and virulence in the citrus postharvest pathogen *Penicillium digitatum*. Fungal Genet. Biol. 67**,** 58-70.

Gandía, M., Xu, S., Font, C., Marcos, J.F., 2016. Disruption of *ku70* involved in non-homologous end-joining facilitates homologous recombination but increases temperature sensitivity in the phytopatogenic fungus *Penicillium digitatum*. Fungal Biol. 120**,** 317-323.

Garrigues, S., Gandía, M., Marcos, J.F., 2016. Occurrence and function of fungal antifungal proteins: a case study of the citrus postharvest pathogen *Penicillium digitatum*. Appl. Microbiol. Biotech. 100**,** 2243-2256.

Garrigues, S., Gandía, M., Popa, C., Borics, A., Marx, F., Coca, M., Marcos, J.F., Manzanares, P., 2017. Efficient production and characterization of the novel and highly active antifungal protein AfpB from *Penicillium digitatum*. Sci Rep 7**,** 14663.

Hamer, J.E., Timberlake, W.E., 1987. Functional organization of the *Aspergillus nidulans trpC* promoter. Mol. Cell. Biol. 7**,** 2352-2359.

652 Harries, E., Gandia, M., Carmona, L., Marcos, J.F., 2015. The *Penicillium*
653 *digitatum* protein *O*-mannosyltransferase Pmt2 is required for cell wall
654 integrity, conidiogenesis, virulence and sensitivity to the antifungal
655 peptide PAF26. Mol. Plant Pathol. 16**,** 748-761.
656 Holmes, G.J., Eckert, J.W., 1999. Sensitivity of *Penicillium digitatum* and *P.*
657 *italicum* to postharvest citrus fungicides in California. Phytopathology 89**,**
658 716-721.
659 Kahl, L.J., Endy, D., 2013. A survey of enabling technologies in synthetic
660 biology. J. Biol Eng. 7**,** 13.
661 Khang, C.H., Park, S.Y., Lee, Y.H., Kang, S.C., 2005. A dual selection based,
662 targeted gene replacement tool for *Magnaporthe grisea* and *Fusarium*
663 *oxysporum*. Fungal Genet. Biol. 42**,** 483-492.
664 Khang, C.H., Park, S.Y., Rho, H.S., Lee, Y.H., Kang, S., 2006. Filamentous
665 fungi (*Magnaporthe grisea* and *Fusarium oxysporum*). Meth. Mol. Biol.
666 344**,** 403-420.
667 Ma, H., Sun, X., Wang, M., Gai, Y., Chung, K.R., Li, H., 2016. The citrus
668 postharvest pathogen *Penicillium digitatum* depends on the PdMpkB
669 kinase for developmental and virulence functions. Int. J. Food Microbiol.
670 236**,** 167-176.
671 Marcet-Houben, M., Ballester, A.R., de la Fuente, B., Harries, E., Marcos, J.F.,
672 González-Candelas, L., Gabaldón, T., 2012. Genome sequence of the
673 necrotrophic fungus *Penicillium digitatum*, the main postharvest
674 pathogen of citrus. BMC Genomics 13**,** 646.
675 Mattern, D.J., Valiante, V., Unkles, S.E., Brakhage, A.A., 2015. Synthetic
676 biology of fungal natural products. Front. Microbiol. 6**,** 775.
677 Mazodier, P., Cossart, P., Giraud, E., Gasser, F., 1985. Completion of the
678 nucleotide sequence of the central region of Tn5 confirms the presence
679 of three resistance genes. Nuc. Acids Res. 13**,** 195-205.
680 Meyer, V., Andersen, M.R., Brakhage, A.A., Braus, G.H., Caddick, M.X., Cairns,
681 T.C., de Vries, R.P., Haarmann, T., Hansen, K., Hertz-Fowler, C.,
682 Krappmann, S., Mortensen, U.H., Peñalva, M.A., Ram, A.F.J., Head,
683 R.M., 2016a. Current challenges of research on filamentous fungi in
684 relation to human welfare and a sustainable bio-economy: a white paper.
685 Fungal Biol. Biotechnol. 3**,** 6.
686 Meyer, V., Nevoigt, E., Wiemann, P., 2016b. The art of design. Fungal Genet.
687 Biol. 89**,** 1-2.
688 Michielse, C.B., Hooykaas, P.J.J., van den Hondel, C.A.M.J., Ram, A.F.J.,
689 2005. *Agrobacterium* -mediated transformation as a tool for functional
690 genomics in fungi. Curr. Genet. 48**,** 1-17.
691 Mullins, E.D., Chen, X., Romaine, P., Raina, R., Geiser, D.M., Kang, S., 2001.
692 *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: An
693 efficient tool for insertional mutagenesis and gene transfer.
694 Phytopathology 91**,** 173-180.
695 Paddon, C.J., Keasling, J.D., 2014. Semi-synthetic artemisinin: A model for the
696 use of synthetic biology in pharmaceutical development. Nat. Rev.
697 Microbiol. 12**,** 355-367.
698 Palou, L., *Penicillium digitatum*, *Penicillium italicum* (Green Mold, Blue Mold), in:
699 Bautista-Baños, S., (Eds.), Postharvest Decay: Control Strategies, 2014,
700 pp. 45-102.

Pall, M.L., Brunelli, J.P., 1993. A series of six compact fungal transformation vectors containing polylinkers with multiple unique restriction sites. Fungal Genet Newsl 40**,** 22.

Patron, N.J., Orzaez, D., Marillonnet, S., Warzecha, H., Matthewman, C., Youles, M., Raitskin, O., Leveau, A., Farré, G., Rogers, C., Smith, A., Hibberd, J., Webb, A.A.R., Locke, J., Schornack, S., Ajioka, J., Baulcombe, D.C., Zipfel, C., Kamoun, S., Jones, J.D.G., Kuhn, H., Robatzek, S., Van Esse, H.P., Sanders, D., Oldroyd, G., Martin, C., Field, R., O'Connor, S., Fox, S., Wulff, B., Miller, B., Breakspear, A., Radhakrishnan, G., Delaux, P.M., Loqué, D., Granell, A., Tissier, A., Shih, P., Brutnell, T.P., Quick, W.P., Rischer, H., Fraser, P.D., Aharoni, A., Raines, C., South, P.F., Ané, J.M., Hamberger, B.R., Langdale, J., Stougaard, J., Bouwmeester, H., Udvardi, M., Murray, J.A.H., Ntoukakis, V., Schäfer, P., Denby, K., Edwards, K.J., Osbourn, A., Haseloff, J., 2015. Standards for plant synthetic biology: A common syntax for exchange of DNA parts. New Phytologist 208**,** 13-19.

Pérez-González, A., Kniewel, R., Veldhuizen, M., Verma, H.K., Navarro-Rodríguez, M., Rubio, L.M., Caro, E., 2017. Adaptation of the GoldenBraid modular cloning system and creation of a toolkit for the expression of heterologous proteins in yeast mitochondria. BMC Biotechnol. 17**,** 80.

Perez-Nadales, E., Almeida Nogueira, M.F., Baldin, C., Castanheira, S., El Ghalid, M., Grund, E., Lengeler, K., Marchegiani, E., Mehrotra, P.V., Moretti, M., Naik, V., Oses-Ruiz, M., Oskarsson, T., Schäfer, K., Wasserstrom, L., Brakhage, A.A., Gow, N.A.R., Kahmann, R., Lebrun, M.H., Perez-Martin, J., Di Pietro, A., Talbot, N.J., Toquin, V., Walther, A., Wendland, J., 2014. Fungal model systems and the elucidation of pathogenicity determinants. Fungal Genet. Biol. 70**,** 42-67.

Polli, F., Meijrink, B., Bovenberg, R.A.L., Driessen, A.J.M., 2016. New promoters for strain engineering of *Penicillium chrysogenum*. Fungal Genet. Biol. 89**,** 62-71.

Punt, P.J., Dingemanse, M.A., Kuyvenhoven, A., Soede, R.D.M., Pouwels, P.H., van den Hondel, C.A.M.J.J., 1990. Functional elements in the promoter region of the *Aspergillus nidulans gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase. Gene 93**,** 101-109.

Ramamoorthy, V., Zhao, X.H., Snyder, A.K., Xu, J.R., Shah, D.M., 2007. Two mitogen-activated protein kinase signalling cascades mediate basal resistance to antifungal plant defensins in *Fusarium graminearum*. Cell. Microbiol. 9**,** 1491-1506.

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

Sarrion-Perdigones, A., Palací, J., Granell, A., Orzaez, D., Design and construction of multigenic constructs for plant biotechnology using the GoldenBraid cloning strategy. Meth. Mol. Biol., Vol. 1116, 2014, pp. 133-151.

Sarrion-Perdigones, A., Vazquez-Vilar, M., Palací, J., Castelijns, B., Forment, J., Ziarsolo, P., Blanca, J., Granell, A., Orzaez, D., 2013. GoldenBraid

2.0: A comprehensive DNA assembly framework for plant synthetic biology. Plant Physiol. 162, 1618-1631.

Segorbe, D., Di Pietro, A., Pérez-Nadales, E., Turrà, D., 2016. Three *Fusarium oxysporum* mitogen-activated protein kinases (MAPKs) have distinct and complementary roles in stress adaptation and cross-kingdom pathogenicity. Mol. Plant Pathol. 18, 912-924.

Sonderegger, C., Galgóczy, L., Garrigues, S., Fizil, Á., Borics, A., Manzanares, P., Hegedüs, N., Huber, A., Marcos, J.F., Batta, G., Marx, F., 2016. A *Penicillium chrysogenum*-based expression system for the production of small, cysteine-rich antifungal proteins for structural and functional analyses. Microb. Cell Fact. 15, 192.

Sun, X., Ruan, R., Lin, L., Zhu, C., Zhang, T., Wang, M., Li, H., Yu, D., 2013. Genomewide investigation into DNA elements and ABC transporters involved in imazalil resistance in *Penicillium digitatum*. FEMS Microbiol. Lett. 348, 11-18.

Terfrüchte, M., Joehnk, B., Fajardo-Somera, R., Braus, G.H., Riquelme, M., Schipper, K., Feldbrügge, M., 2014. Establishing a versatile Golden Gate cloning system for genetic engineering in fungi. Fungal Genet. Biol. 62, 1-10.

Vazquez-Vilar, M., Bernabé-Orts, J.M., Fernandez-del-Carmen, A., Ziarsolo, P., Blanca, J., Granell, A., Orzaez, D., 2016. A modular toolbox for gRNA-Cas9 genome engineering in plants based on the GoldenBraid standard. Plant Methods 12, 10.

Vazquez-Vilar, M., Quijano-Rubio, A., Fernández-Del-Carmen, A., Sarrion-Perdigones, A., Ochoa-Fernandez, R., Ziarsolo, P., Blanca, J., Granell, A., Orzaez, D., 2017. GB3.0: a platform for plant bio-design that connects functional DNA elements with associated biological data. Nucleic Acids Res 45, 2196-2209.

Wang, M., Chen, C., Zhu, C., Sun, X., Ruan, R., Li, H., 2014. Os2 MAP kinase-mediated osmostress tolerance in *Penicillium digitatum* is associated with its positive regulation on glycerol synthesis and negative regulation on ergosterol synthesis. Microbiol. Res. 169, 511-521.

Wang, Y., Tian, L., Xiong, D., Klosterman, S.J., Xiao, S., Tian, C., 2016. The mitogen-activated protein kinase gene, VdHog1, regulates osmotic stress response, microsclerotia formation and virulence in *Verticillium dahliae*. Fungal Genet. Biol. 88, 13-23.

Weber, E., Engler, C., Gruetzner, R., Werner, S., Marillonnet, S., 2011. A modular cloning system for standardized assembly of multigene constructs. PLoS ONE 6, e16765.

Zhang, T., Sun, X., Xu, Q., González-Candelas, L., Li, H., 2013a. The pH signaling transcription factor PacC is required for full virulence in *Penicillium digitatum*. Appl. Microbiol. Biotech. 97, 9087-9098.

Zhang, T., Sun, X., Xu, Q., Zhu, C., Li, Q., Li, H., 2013b. *PdSNF1*, a sucrose non-fermenting protein kinase gene, is required for *Penicillium digitatum* conidiation and virulence. Appl. Microbiol. Biotech. 97, 5433-5445.

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

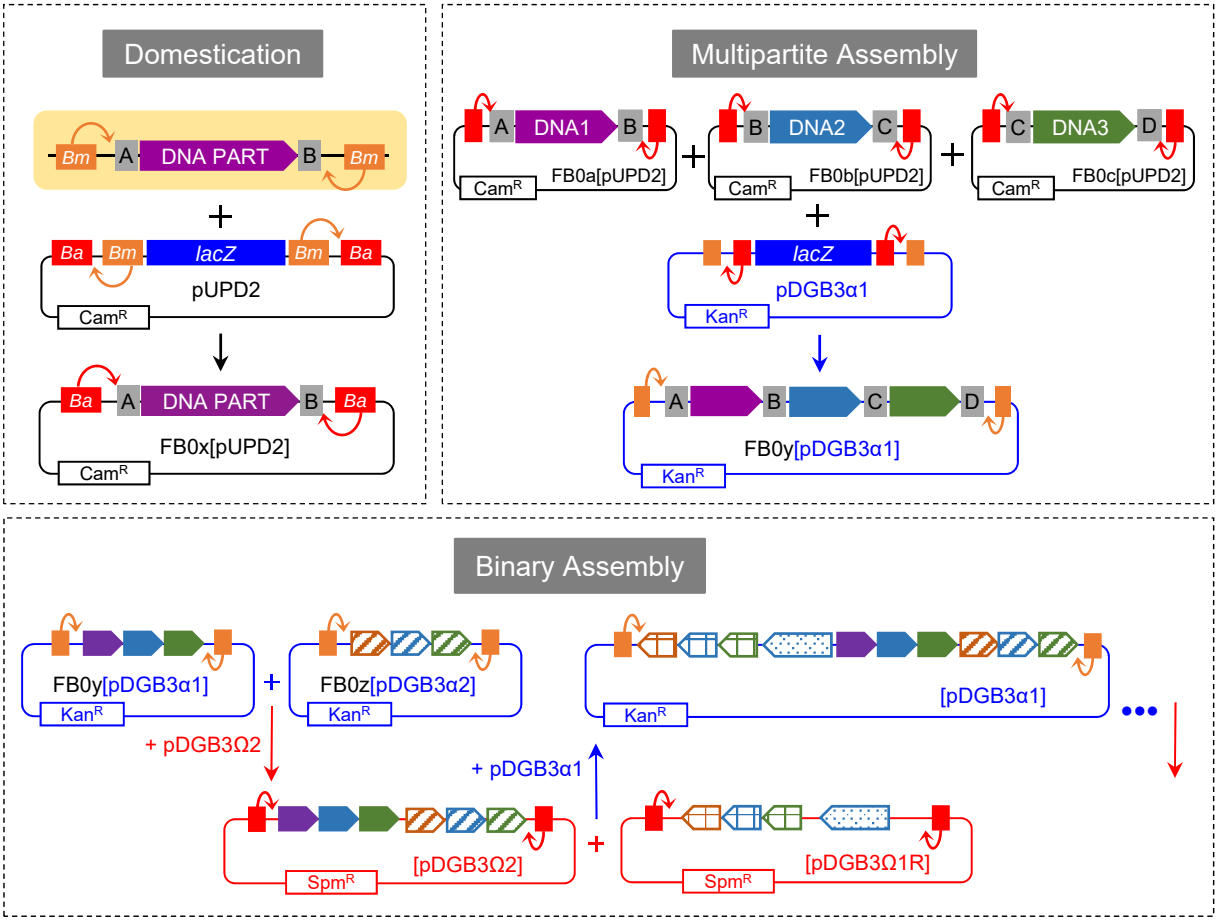


Figure 2 (Hernanz-Koers *et al.*, 2018)

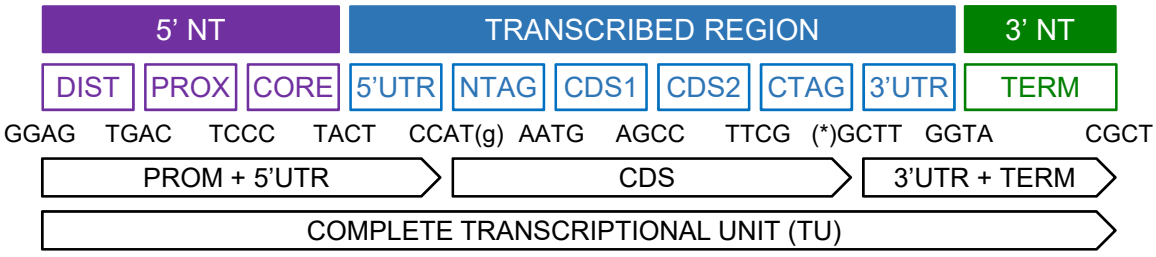


Figure 3 (Hernanz-Koers *et al.*, 2018)

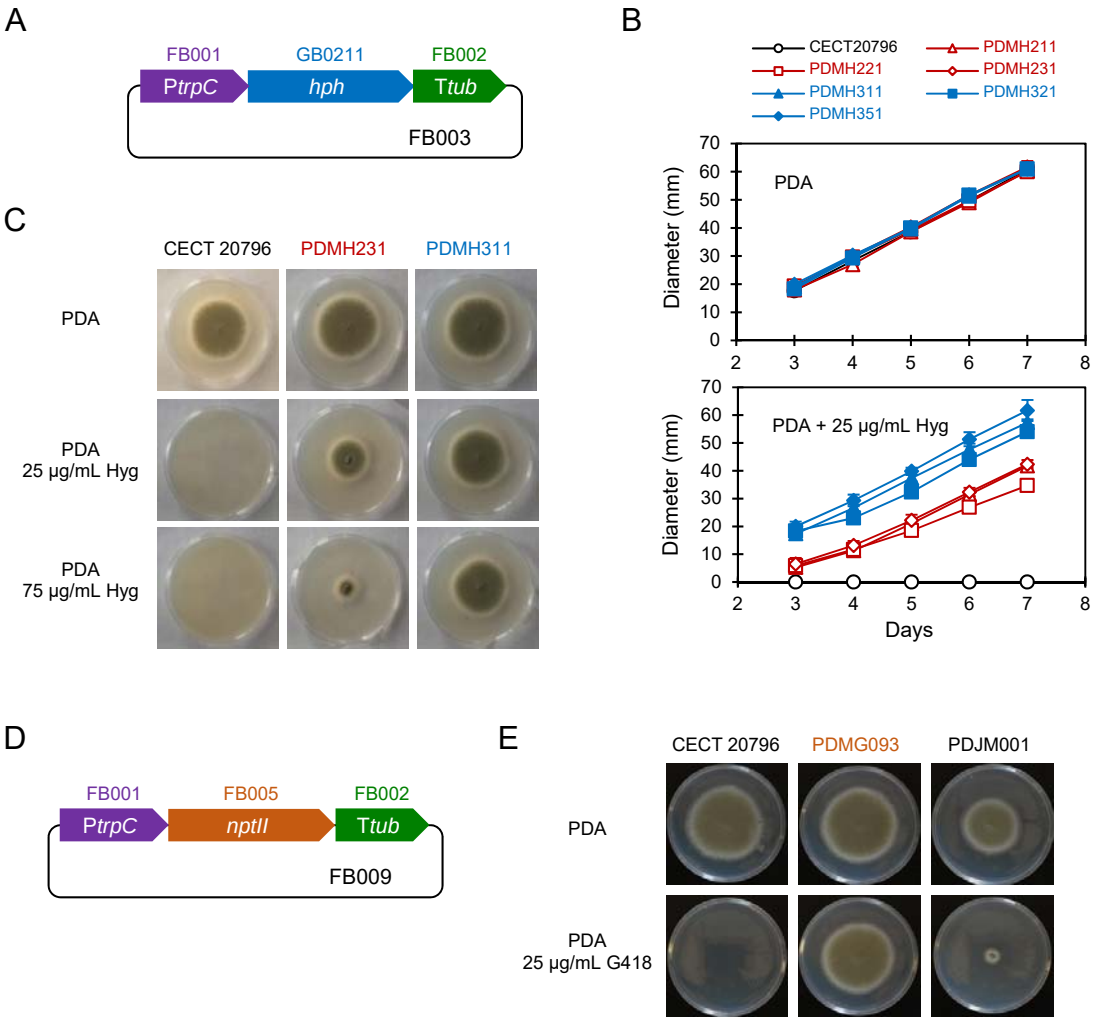


Figure 4 (Hernanz-Koers *et al*., 2018)

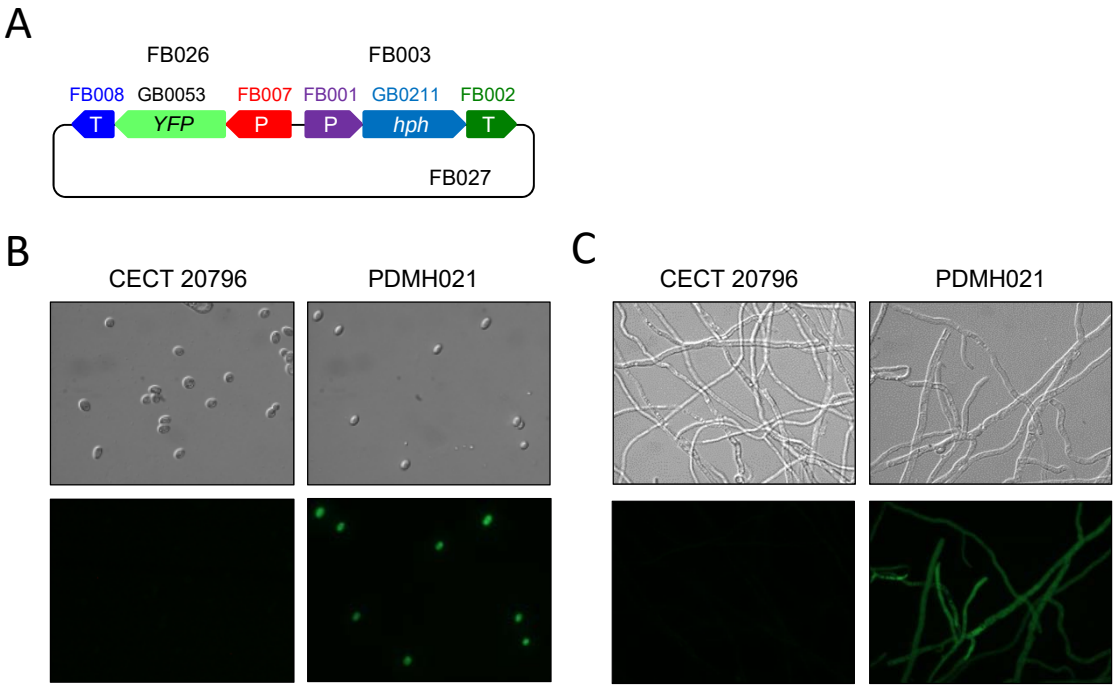


Figure 5 (Hernanz-Koers *et al*., 2018)

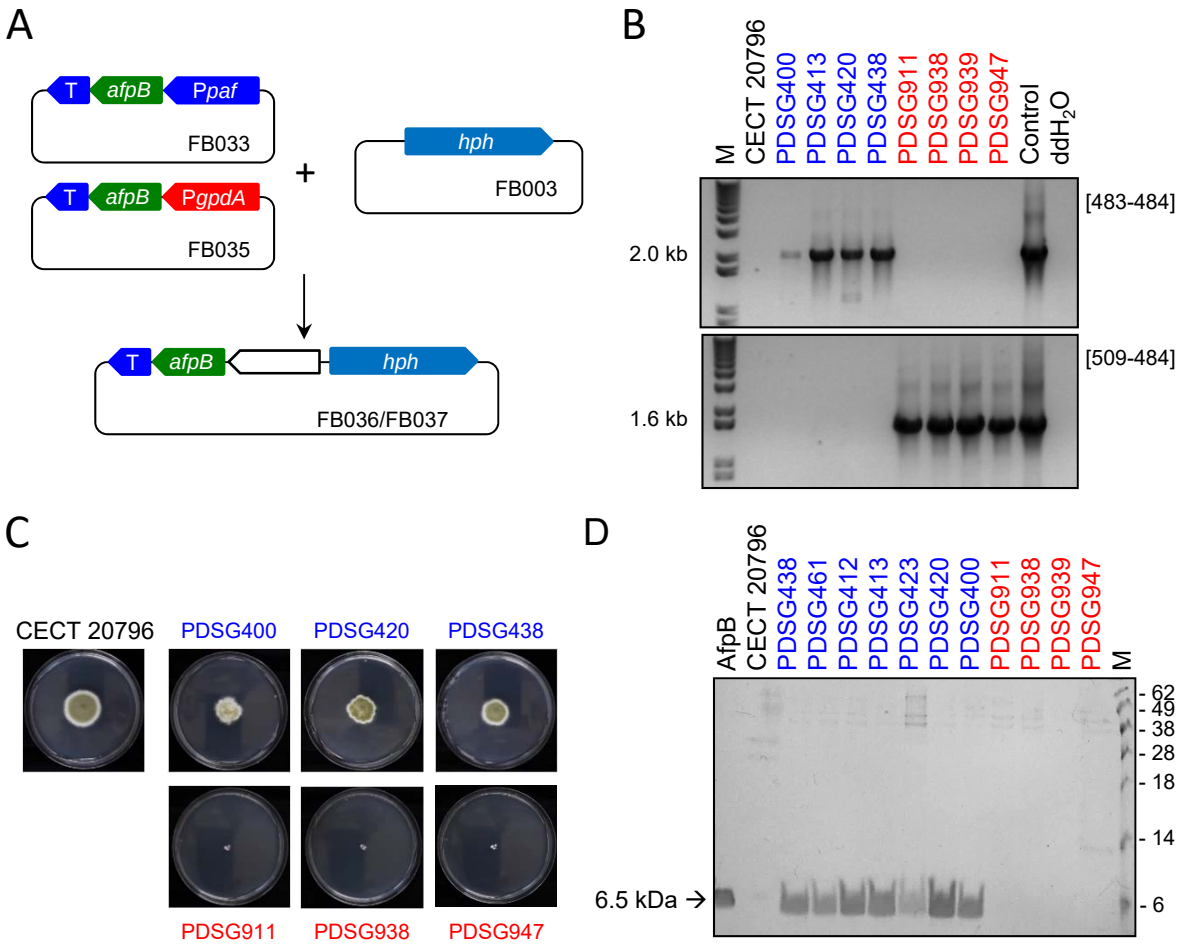
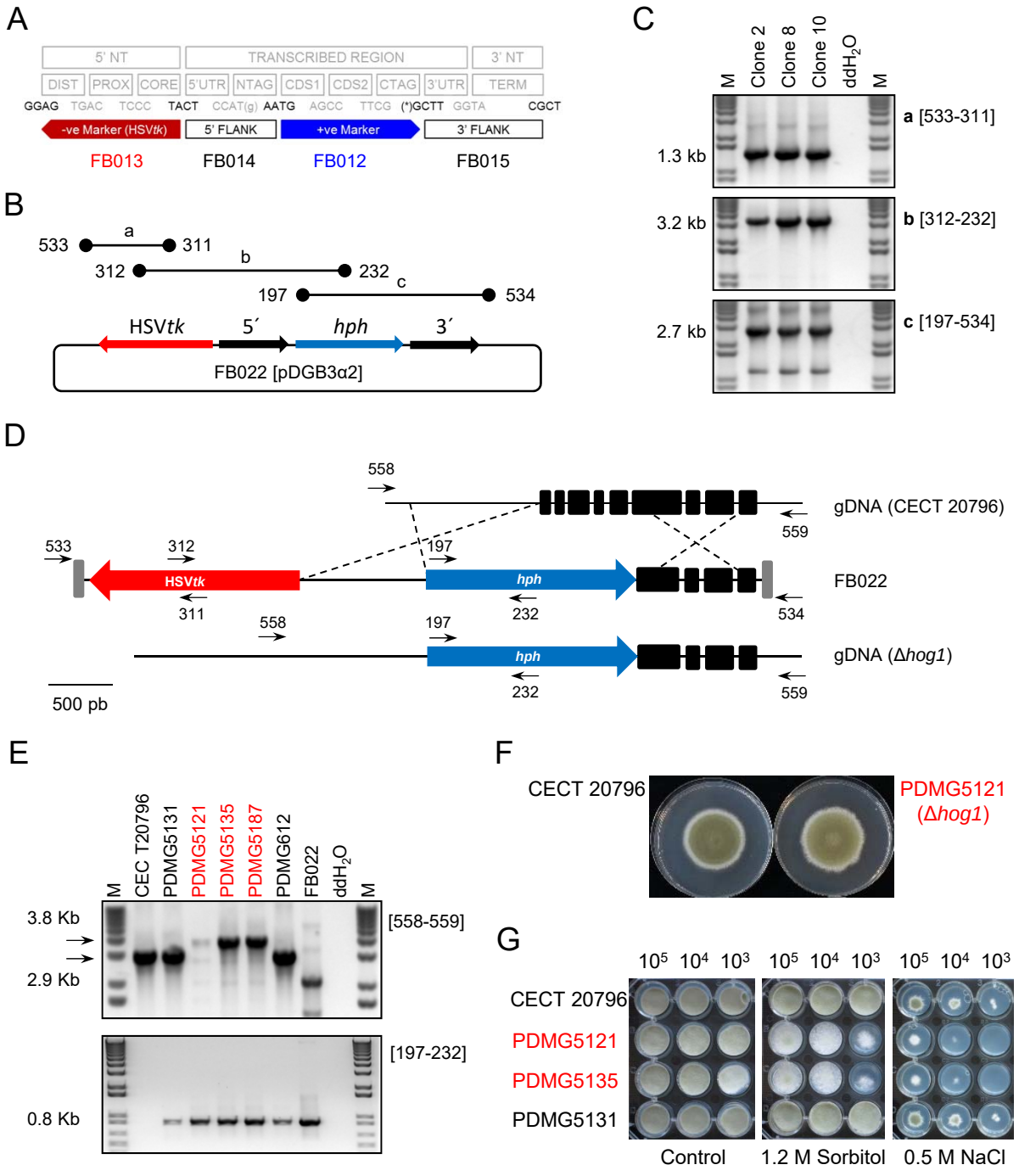
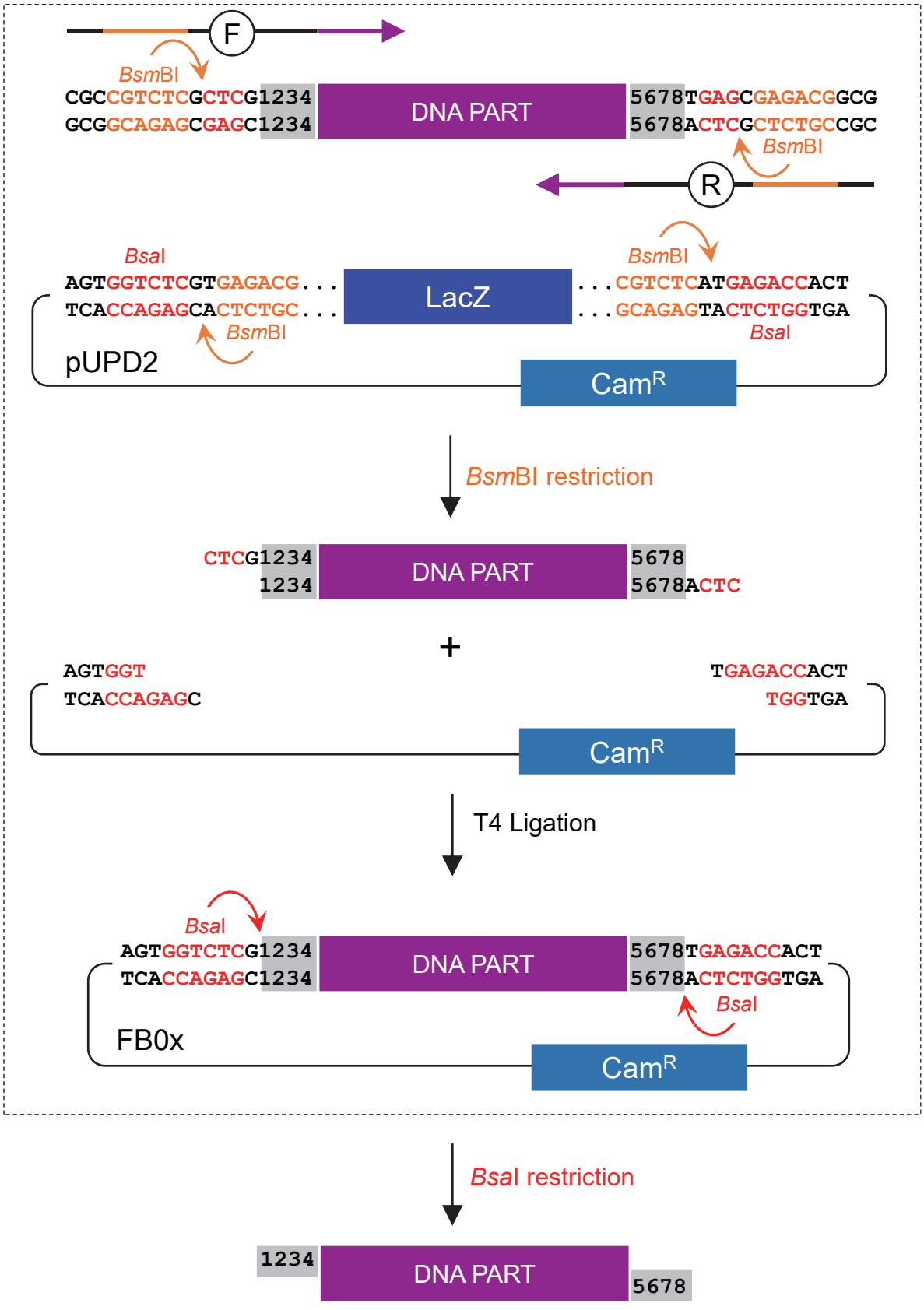


Figure 6 (Hernanz_Koers *et al*., 2017)

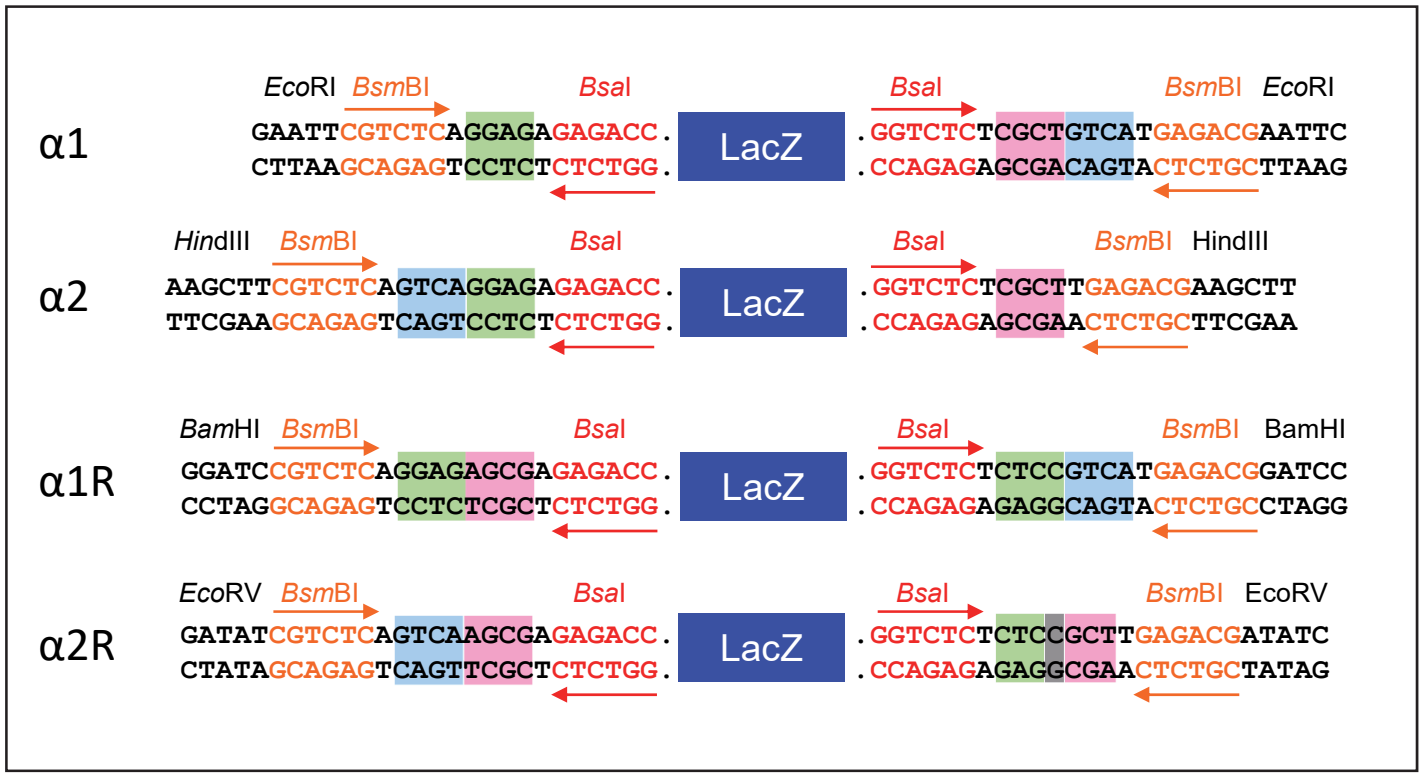


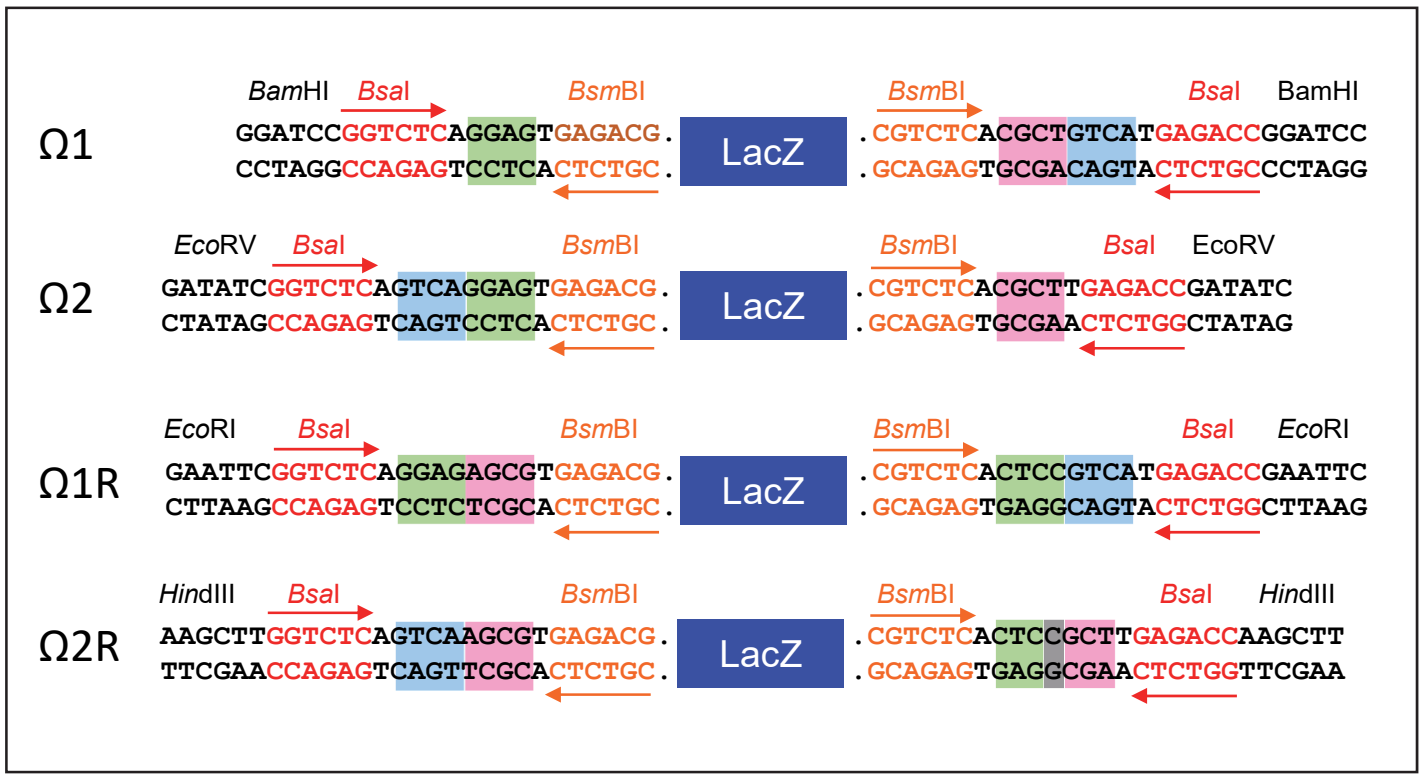
Supplemental Figure S1 (Hernanz-Koers *et al.*, 2018)



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 *Bsm*BI 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 *Bsm*BI 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 *Bsa*I to produce a dsDNA with “1234” and “5678” flanking overhangs, which will be used in the subsequent “Multipartite Assembly” step (Figure 1). *Bsm*BI and *Bsa*I recognition sequences are depicted in orange and red, respectively, in the DNA sequence. Outside the *Bsa*I sites, plasmid pUPD2 also contains *Btg*ZI 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., 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/).

Supplemental Figure S2 (Hernanz-Koers *et al.*, 2018)





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, *Hin*dIII, *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/).