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Development of a FungalBraid *Penicillium expansum*-based expression system for the production of antifungal proteins in fungal biofactories

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Summary

Fungal antifungal proteins (AFPs) have attracted attention as novel biofungicides. Their exploitation requires safe and cost-effective producing biofactories. Previously, *Penicillium chrysogenum* and *Penicillium digitatum* produced recombinant AFPs with the use of a *P. chrysogenum*-based *expression system that consisted of the paf* gene promoter, signal peptide (SP)-pro sequence and terminator. Here, the regulatory elements of the *afpA* gene encoding the highly produced PeAfpA from *Penicillium expansum* were developed as an expression system for AFP production through the FungalBraid platform. The *afpA* cassette was tested to produce PeAfpA and *P. digitatum* PdAfpB in *P. chrysogenum* and *P. digitatum*, and its efficiency was compared to that of the *paf* cassette. Recombinant PeAfpA production was only achieved using the *afpA* cassette, being *P. chrysogenum* a more efficient biofactory than *P. digitatum*. Conversely, *P. chrysogenum* only produced PdAfpB under the control of the *paf* cassette. In *P. digitatum*, both expression systems allowed PdAfpB production, with the *paf* cassette resulting in higher protein yields. Interestingly, these results did not correlate with the performance of both promoters in a luciferase reporter system. In conclusion, AFP production is a complex outcome that depends on the regulatory sequences driving *afp* expression, the fungal biofactory and the AFP sequence.

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Introduction

Antifungal proteins (AFPs) secreted by filamentous ascomycetes have lately gained attention as biofungicides since they specifically inhibit fungal growth without affecting plant or mammalian cell viability (Vila *et al*., 2001; Szappanos *et al*., 2005, 2006; Palicz *et al*., 2013). AFPs are small cationic cysteine-rich proteins (CRPs) that form three or four disulfide bonds and fold into compact tertiary structures, which makes AFPs highly stable against adverse biochemical and biophysical conditions such as pH, temperature and proteolysis (Batta *et al*., 2009). Similar to other CRPs, AFPs contain a conserved γ-core motif (Yount and Yeaman, 2004) and are coded with a signal peptide (SP) at the N-termini that includes a pre-sequence involved in AFP secretion to the extracellular space and a pro-sequence (SP-pro sequence) that has been predicted to inactivate the protein until cleavage (Marx *et al*., 1995). AFPs exhibit potent antifungal activity and different mechanisms of action against opportunistic human, animal, plant and foodborne pathogenic fungi (Marx *et al*., 2008; Hegedüs and Marx, 2013; Delgado *et al*., 2016; Tóth *et al*., 2020a; Czajlik *et al*., 2021; Martínez-Culebras *et al*., 2021).

The first identified AFPs were the one produced by *Aspergillus giganteus* (Nakaya *et al*., 1990) and the so-called PAF protein secreted by *Penicillium chrysogenum* (Marx *et al*., 1995). Both are abundantly secreted, with yields above 50 mg l^{-1}. This is also the case of the protein PeAfpA from *Penicillium expansum*, with purification yields reaching up to 125 mg l^{-1} (Garrigues *et al*., 2018). By contrast, the AFPs from *Neosartorya* (*Aspergillus*) *fischeri*, NFAP and NFAP2, are produced in modest amounts (Kovács *et al*., 2011; Tóth *et al*., 2016), while PdAfpB from *Penicillium digitatum* and PAFB from *P. chrysogenum* remained undetectable in the culture medium although their encoding genes were transcribed at high levels (Garrigues *et al*., 2016; Huber *et al*., 2018).

The potential use of AFPs as novel biocidal compounds in crop and postharvest protection (Vila *et al*., 2001; Theis *et al*., 2005; Barakat, 2014; Garrigues *et al*., 2018, 2020; Delgado *et al*., 2019; Tóth *et al*., 2020a, 2020b; Gandía *et al*., 2021), medicine (Garrigues *et al*., 2018; Tóth *et al*., 2018; Kovács *et al*., 2019; Holzknecht *et al*., 2020) and food preservation (Delgado *et al*., 2015; Martínez-Culebras *et al*., 2021) has been extensively reported, but mainly under laboratory conditions. Undoubtedly, further exploitation of AFPs requires safe, efficient and economic biofactories for their production. *P. chrysogenum* was demonstrated to serve as an efficient fungal expression factory for AFPs. The expression of recombinant AFPs was achieved with the use of a *P. chrysogenum*-based *expression system, the paf* cassette, which consisted of the strong *paf* gene promoter (P*paf*), *paf* SP-pro sequence for protein processing and secretion, and terminator (T*paf*) (Sonderegger *et al*., 2016). This approach allowed the overexpression of high amounts of PAF, PAF mutants, NFAP and NFAP2 (Sonderegger *et al*., 2016, 2018; Tóth *et al*., 2018), *P. expansum* PeAfpB and PeAfpC (Garrigues *et al*., 2018) and *P. chrysogenum* PAFB (Huber *et al*., 2018) and PAFC (Holzknecht *et al*., 2020). The versatility of this system was demonstrated with the successful expression of PAF and PdAfpB in *P. digitatum* (Sonderegger *et al*., 2016; Garrigues *et al*., 2017).

FungalBraid (FB) is a synthetic biology modular cloning platform for the assembly and exchange of DNA elements tailored to fungal biotechnology and adapted from the GoldenBraid (GB) system developed for plants (Sarrión-Perdigones *et al*., 2011; Vázquez-Vilar *et al*., 2017, 2020; Hernanz-Koers *et al*., 2018). Moreover, both GB and FB systems are fully compatible and allow the exchange of suitable DNA parts between plants and fungi. We had previously adapted the DNA elements of the *paf* cassette to FB to facilitate the straightforward cloning and efficient production of AFPs and rationally designed variants (Heredero *et al*., 2018; Hernanz-Koers *et al*., 2018). The *paf* cassette was also successful to homologously produce proteins different from AFPs such as Sca, an anionic *P. digitatum* CRP, in high yields (Garrigues *et al*., 2020).

Since PeAfpA is a highly secreted protein that reaches yields above 100 mg l^{-1} in its wild-type producing fungus, regulatory elements of *afpA* gene provide an excellent opportunity to develop a *P. expansum*-based *expression system for the production of AFPs and likely other CRPs. In this work, we have evaluated the feasibility of the afpA* gene promoter and terminator sequences to drive the expression of AFPs. This *afpA* cassette, together with the previously described *paf* cassette, have been assessed for PeAfpA and PdAfpB production, using their corresponding native signal peptide (SP)-pro sequences, in *P. chrysogenum* and *P digitatum*. Finally, the strength of *afpA* and *paf* promoters was tested and compared in a luciferase-based reporter system.

Results

Development of a FungalBraid P. expansum*-based* expression system

The FB system follows the standards of synthetic biology and enables reusability of genetic parts and direct comparison of experiments among different fungal species. In this study, we used different already available FB elements and generated others to compare the regulatory elements of the *afpA* and *paf* genes to produce PeAfpA and PdAfpB in two fungal biofactories, *P. chrysogenum* and *P. digitatum*.

For the development of the *afpA* cassette, three genetic elements were domesticated: the *afpA* promoter (P*afpA*) and terminator (T*afpA*) sequences from *P. expansum* and the coding sequence (CDS) from the *afpA* gene including its own SP-pro sequence (Table 1). In order to compare the *P. expansum*-based expression system with the previously described *paf* cassette (Sonderegger *et al*., 2016), we used the already available elements P*paf* and T*paf* together with the *afpB* gene sequence from *P. digitatum* (Hernanz-Koers *et al*., 2018). Former studies from our group showed that *P. digitatum* produced up to 20 mg l^{-1} of PdAfpB when expressed under the control of P*paf* and T*paf* (Garrigues *et al*., 2017).

To test and compare the production of PeAfpA and PdAfpB under the control of either P*afpA* and T*afpA*, or P*paf* and T*paf* sequences, FB multipartite and binary assemblies were successfully performed, and the binary vectors needed for the final transformation of either *P. chrysogenum* or *P. digitatum* were generated (Table 1; Fig. 1; see experimental procedures for further details).

Table 1. GoldenBraid (GB) and FungalBraid (FB) elements used and FB vectors generated in this study.

Code	Genetic Element(s)	GB Plasmid	References
GB0096	*Luciferase (luc)*	pUPD	GB website[a]
GB3458	*3a1_AtS/MAR10* insulator sequence	pDGB3α1	Pérez-González *et al.* (2019)
FB002	T*tub*	pUPD2	Hernanz-Koers *et al.* (2018)
FB003	P*trpC*::*hph*::T*tub*	pDGB3α2	Hernanz-Koers *et al.* (2018)
FB007	P*gpdA*	pUPD2	Hernanz-Koers *et al.* (2018)
FB009	P*trpC*::*nptII*::T*tub*	pDGB3α2	Hernanz-Koers *et al.* (2018)
FB029	P*paf*	pUPD2	Hernanz-Koers *et al.* (2018)
FB030	T*paf*	pUPD2	Hernanz-Koers *et al.* (2018)
FB031	*afpB*	pUPD2	Hernanz-Koers *et al.* (2018)
FB033	P*paf*::*afpB*::T*paf*	pDGB3α1R	Hernanz-Koers *et al.* (2018)
FB036	P*paf*::*afpB*::T*paf*(←)::P*trpC*::*hph*::T*tub*(→)	pDGB3Ω1	Hernanz-Koers *et al.* (2018)
FB107	P*afpA*	pUPD2	This work
FB108	*afpA*	pUPD2	This work
FB109	T*afpA*	pUPD2	This work
FB112	P*afpA*::*afpA*::T*afpA*	pDGB3α1R	This work
FB114	P*afpA*::*afpA*::T*afpA*(←)::P*trpC*::*hph*::T*tub*(→)	pDGB3Ω1	This work
FB115	P*afpA*::*afpA*::T*afpA*(←)::P*trpC*::*nptII*::T*tub*(→)	pDGB3Ω1	This work
FB116	P*paf*::*afpB*::T*paf*(←)::P*trpC*::*nptII*::T*tub*(→)	pDGB3Ω1	This work
FB146	P*paf*::*afpA*::T*paf*	pDGB3α1R	This work
FB158	P*paf*::*afpA*::T*paf*(←)::P*trpC*::*hph*::T*tub*(→)	pDGB3Ω1	This work
FB159	P*paf*::*afpA*::T*paf*(←)::P*trpC*::*nptII*::T*tub*(→)	pDGB3Ω1	This work
FB230	P*afpA*::*afpB*::T*afpA*	pDGB3α1R	This work
FB244	P*afpA*::*afpB*::T*afpA*(←)::P*trpC*::*hph*::T*tub*(→)	pDGB3Ω1	This work
FB245	P*afpA*::*afpB*::T*afpA*(←)::P*trpC*::*nptII*::T*tub*(→)	pDGB3Ω1	This work
FB258	P*paf*::*luc*::T*trpC*	pDGB3α2	This work
FB259	P*afpA*::*luc*::T*trpC*	pDGB3α2	This work
FB261	*AtS/MAR10*(→)::P*paf*::*luc*::T*trpC*(→)	pDGB3Ω2	This work
FB262	*AtS/MAR10*(→)::P*afpA*::*luc*::T*trpC*(→)	pDGB3Ω2	This work
FB310	*Nanoluciferase* (*Nanoluc*)	pUPD2	This work
FB312	P*gpdA*::*Nanoluc*::T*tub*	pDGB3α1	This work
FB316	P*gpdA*::*Nanoluc*::T*tub*(→)::P*trpC*::*nptII*::T*tub*(→)	pDGB3Ω1	This work
FB323	FB316(→)::*AtS/MAR10*(→)::P*paf*::*luc*::T*trpC*(→)	pDGB3α1	This work
FB324	FB316(→)::*AtS/MAR10*(→)::P*afpA*::*luc*::T*trpC*(→)	pDGB3α1	This work

Glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*); neomycin phosphotransferase gene (*nptII*) conferring geneticin resistance; hygromycin-B-phosphotransferase gene (*hph*); tryptophan biosynthesis protein C gene (*trpC*); β-*tubulin* gene (*tub*).
a. https://gbcloning.upv.es/

PeAfpA production is only achieved using the P. expansum*-based* expression system

We used the *afpA* cassette and the *paf* cassette for recombinant production of PeAfpA in *P. chrysogenum* (Table 1; Fig. 2A). Several *P. chrysogenum* positive independent transformants (Fig. S1) were obtained and protein production was evaluated after 5 days of growth in *P. chrysogenum* minimal medium (PcMM) (Sonderegger *et al.*, 2016). Figure 2B shows the SDS-PAGE and western blot analyses of culture supernatants of seven positive clones from each transformation experiment. For recombinant production under the control of the *afpA* cassette (Fig. 2B, left panel), three out of the seven transformants analysed produced a protein band of apparent molecular mass similar to that of the pure PeAfpA. Moreover, PeAfpA-specific signals were observed in these three supernatants when analysed by western blot (PCMG11522, PCMG11532 and PCMG11552), confirming the production of the protein. On the contrary, for recombinant PeAfpA production under the control of the *paf* cassette (Fig. 2B, right panel), no PeAfpA-producing clones were obtained, suggesting that PeAfpA is not present, or is not present in detectable amounts in supernatants of *P. chrysogenum* transformants obtained under the control of the *paf* promoter and terminator sequences. Analysis of the gene copy number by quantitative PCR (qPCR) of genomic DNA indicated that two of the three PeAfpA-producer clones likely contain two copies of the P*afpA*::*afpA*::T*afpA* construction (PCMG11522 and PCMG11552), while the low producer PCMG11532 contains at least 3 copies (Fig. 2C). Figure 2D shows the growth of the *P. chrysogenum* PeAfpA producing clones, the reference strain *P. chrysogenum* Q176 and the parental *P. chrysogenum* strain used for transformation (Δ*paf*), in potato dextrose agar (PDA) and PcMM media. The radial growth of *P. chrysogenum* transformants was indistinguishable from those of the control strains regardless of the medium, although conidia production of PCMG11532 was significantly lower than that showed by control strains and PCMG11522 and PCMG11552 in PDA,

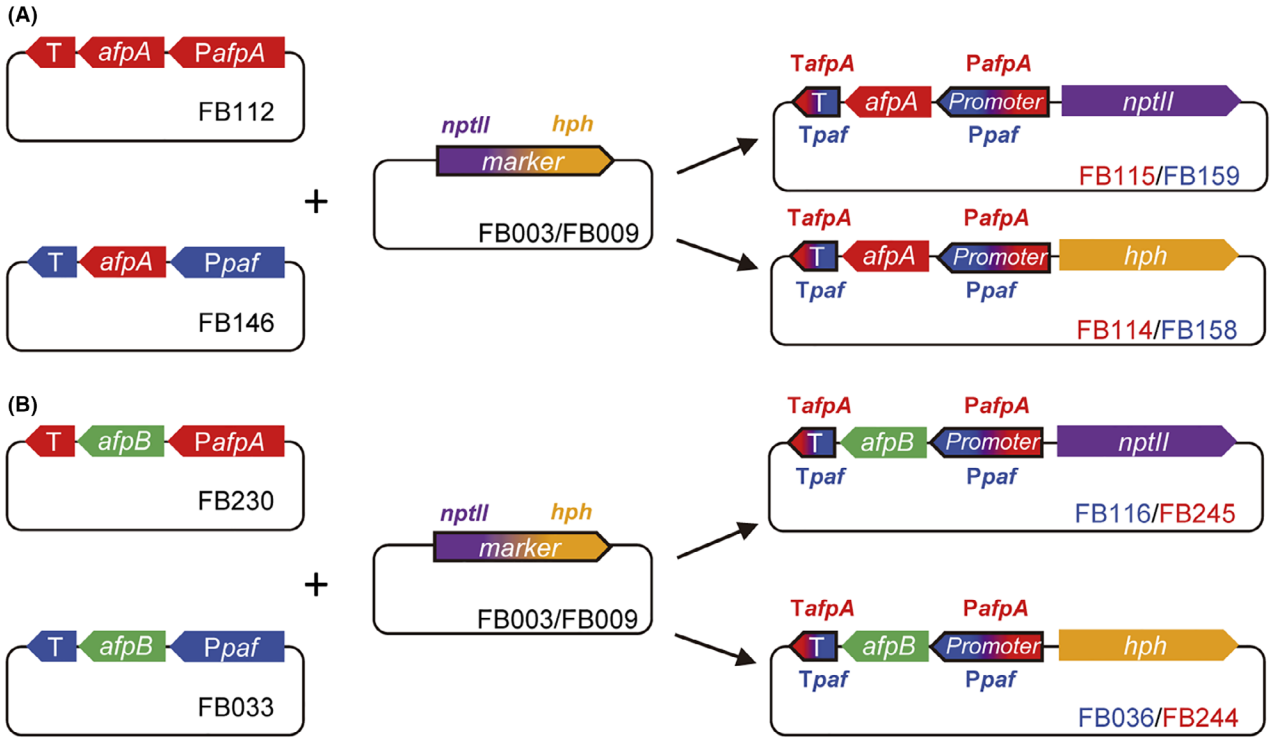


Fig. 1. Schematic diagrams of FungalBraid transcriptional units (TU) and binary vectors for the expression of *afp* genes under the control of either the *afpA* or *paf* cassettes.
A. Binary assembly of TUs FB112 and FB146 with hygromycin (*hph*) resistant marker (FB003) and geneticin (*nptII*) resistant marker (FB009) to obtain the final binary vectors to transform *P. digitatum* (FB114 and FB158) and *P. chrysogenum* (FB115 and FB159) for the production of PeAfpA.
B. Binary assembly of TUs FB230 and FB033 with FB003 and FB009 to obtain the final binary vectors to transform *P. digitatum* (FB036 and FB244) and *P. chrysogenum* (FB116 and FB245) for the production of PdAfpB. FB033 is described in Hernanz-Koers *et al*. (2018).

which was further confirmed by conidia production measurement (Fig. S2). The clone PCMG11552 was selected as the highest recombinant protein producer for further characterization.

Thereafter, we tested both expression systems for the recombinant production of PeAfpA in *P. digitatum* (Fig. 3A). Several *P. digitatum* positive transformants were obtained from each transformation experiment (Fig. S1) and evaluated after 11 days of growth in *P. digitatum* minimal medium (PdMM) (Sonderegger *et al*., 2016). Figure 3B shows that faint PeAfpA-specific signals were immunodetected in three out of six culture supernatants, corresponding to the recombinant production under the control of the *afpA* cassette (PDGL11412, PDGL11432 and PDGL11442). However, no signal was observed in the supernatants corresponding to the *paf* cassette. These results confirm that, in the conditions tested, PeAfpA is only detected in supernatants of transformants obtained under the control of its own regulatory elements. Analysis of the gene copy number revealed that only one copy of the P*afpA*::*afpA*::T*afpA* construction was randomly inserted in these three strains in which mild PeAfpA production was detected (Fig. 3C).

The growth in PDA and PdMM of the *P. digitatum* PeAfpA-producing clones and the parental *P. digitatum* strain used for transformation (CECT 20796) are shown in Fig. 3D. *P. digitatum* transformants did not show phenotypical differences compared to the parental strain in both media.

Comparison of production between recombinant PeAfpA-producing strain P. chrysogenum *PCMG11552 and wild-type* P. expansum *CMP-1*

To compare the level of PeAfpA production, the selected PeAfpA producer PCMG11552 and *P. expansum* CMP-1 were grown in PcMM and, subsequently, analyzed by SDS-PAGE and western blot in a time-course experiment (Fig. 4A). As expected, PeAfpA-specific signals were immunodetected in most of the supernatants evaluated. Both strains produced PeAfpA from 5 to 10 days of growth. Nevertheless, in CMP-1 supernatants, PeAfpA amount increased throughout the experiment, whereas in PCMG11552, the protein reached a maximum at day 7. With the exception of 5-day supernatants, higher quantities of PeAfpA were detected in CMP-1 supernatants.

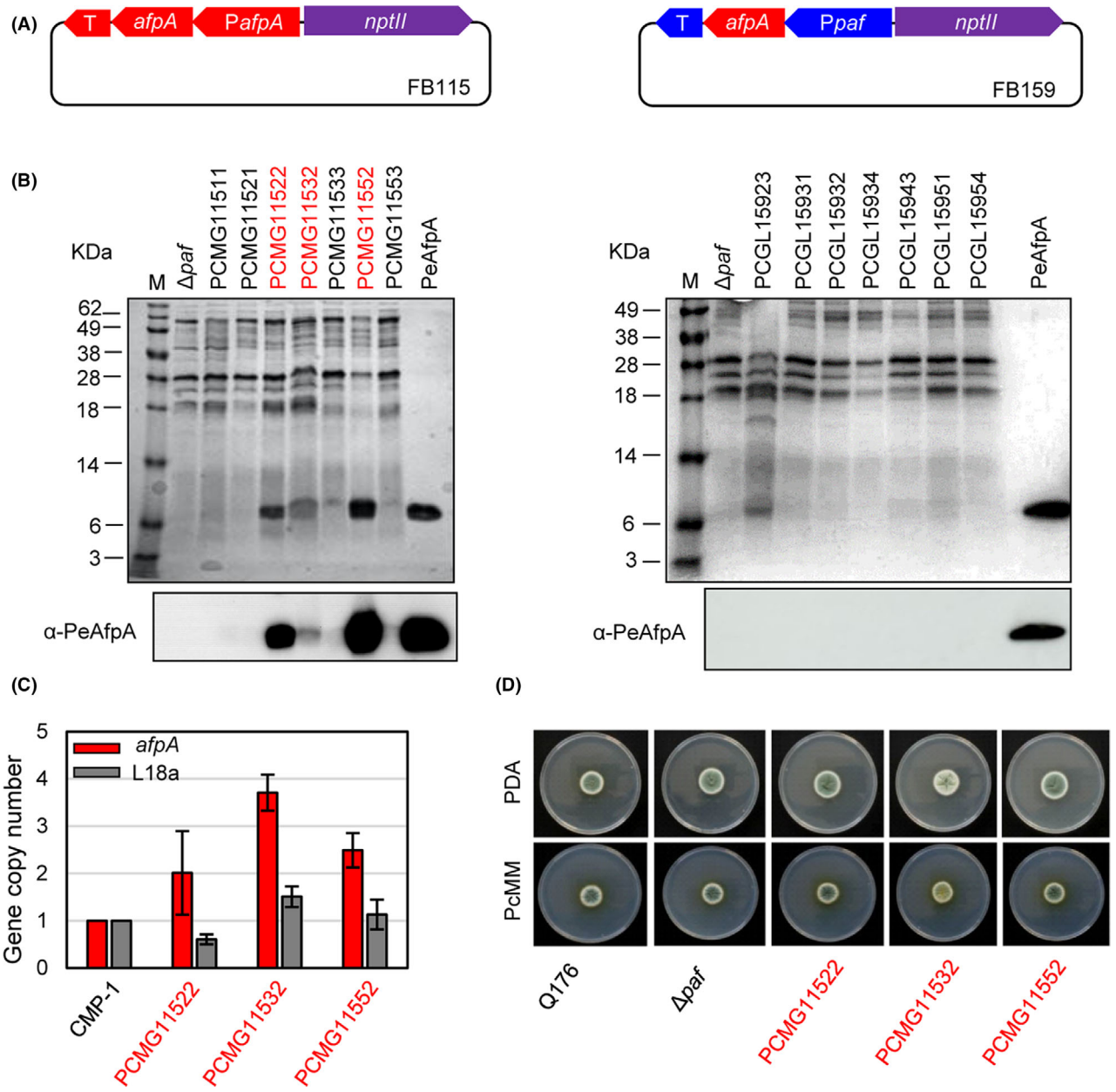


Fig. 2. Analyses of *P. chrysogenum* transformants for PeAfpA production with either the *afpA* or the *paf* cassette.
A. Schematic diagram of the binary vectors FB115 and FB159 used for *P. chrysogenum* transformation.
B. SDS-PAGE (top) and western blot analyses (bottom) of pure PeAfpA (2 μg) and growth supernatants of recombinant strains (10 μl of 10× supernatants loaded per lane) obtained by either FB115 (left) or FB159 (right) transformation. SDS-PAGE analyses were visualized by Coomassie blue staining; M: SeeBlue® Pre-stained protein standard. Immunoblot analyses were performed using specific anti-PeAfpA antibody. Parental strain Δ*paf* was loaded as a negative control. Positive PeAfpA producing strains (PCMG11522, PCMG11532 and PCMG11552) are highlighted in red.
C. Evaluation of *afpA* gene copy number in the different PeAfpA producing strains by qPCR. The Ct signal of *afpA* and L18a was normalized to that of β-*tub*. Results are presented as mean values ± standard deviation (SD) of three technical replicates. Under this experimental design, the resulting gene copy number is expected to be 1 for *afpA* in CMP-1, and ≥ 1 for *afpA* in *P. chrysogenum* transformants.
D. Colony morphology of *P. chrysogenum* PeAfpA producing strains PCMG11522, PCMG11532 and PCMG11552 compared to the wild-type Q176 and the parental strain Δ*paf* after 6 days of growth on PDA and PcMM plates.

Next, we characterized the processing of the recombinant PeAfpA. For this purpose, the protein was purified from PcMM supernatant of PCMG11552 grown for 5 days. After one-step cation-exchange chromatography, pure protein was subjected to peptide mass fingerprinting (PMF). Figure 4B shows a sequence coverage of 49% and the proper

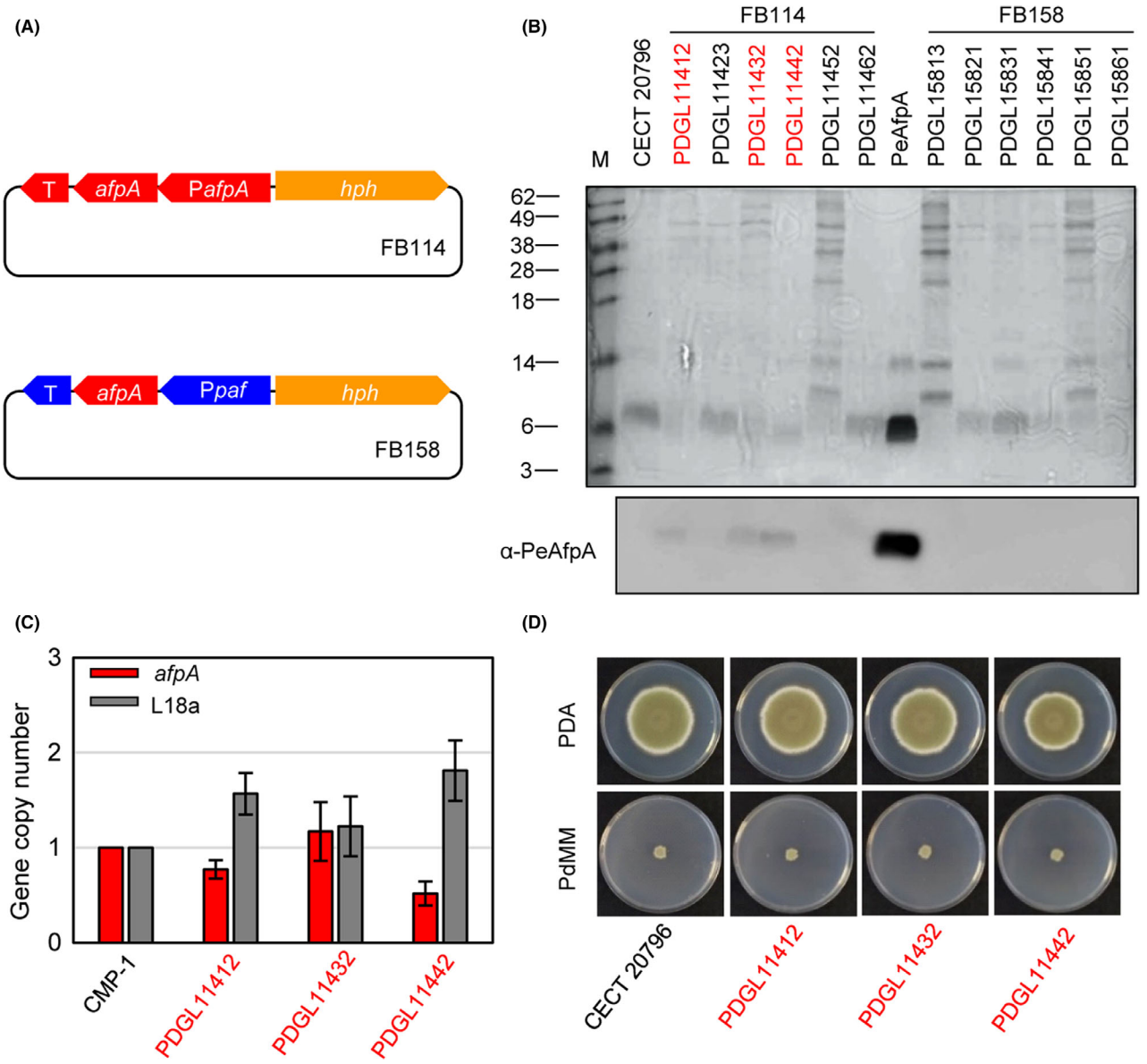
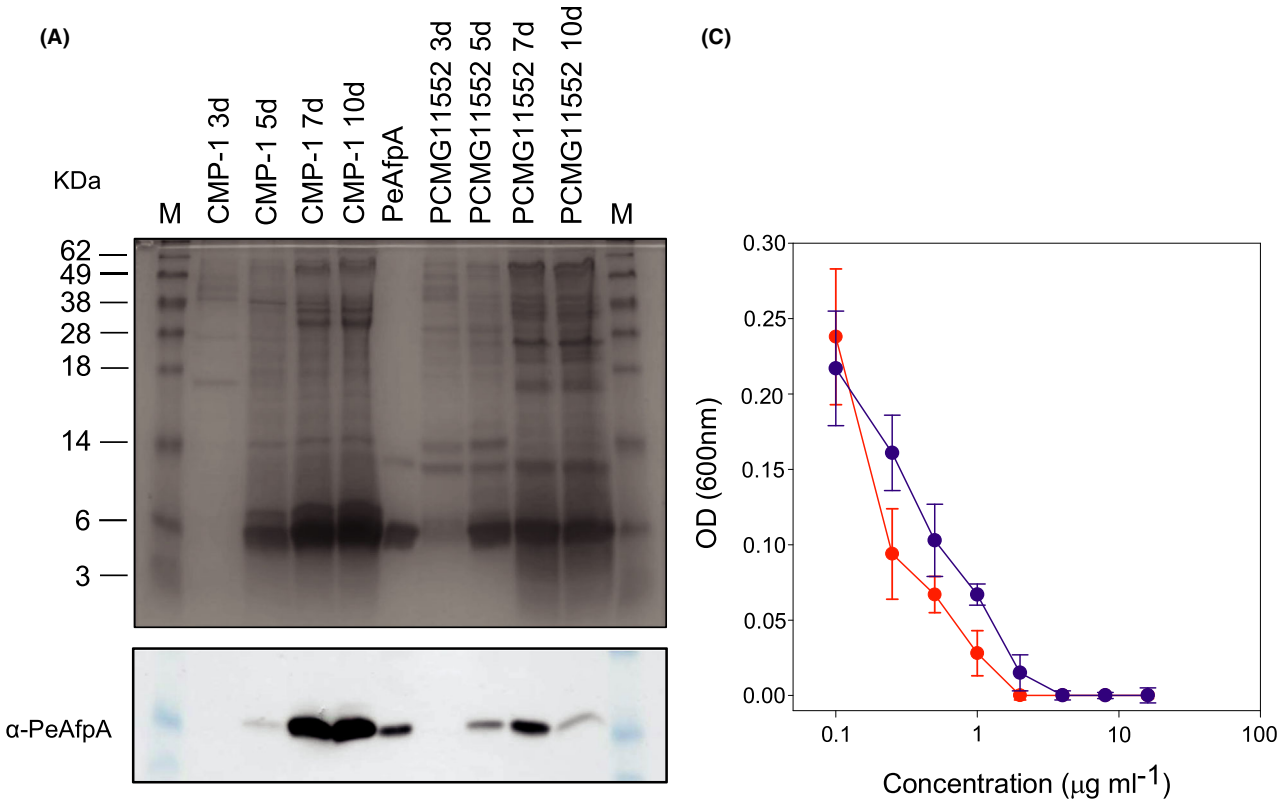


Fig. 3. Analyses of *P. digitatum* transformants for PeAfpA production using either the *afpA* or the *paf* cassette.
A. Schematic diagram of the binary vectors FB114 and FB158 used for *P. digitatum* transformation.
B. SDS-PAGE (top) and western blot analysis (bottom) of pure PeAfpA (2 μg) and growth supernatants of recombinant strains (10 μl of 10× supernatants loaded per lane) obtained by either FB114 or FB158 transformation. SDS-PAGE analyses were visualized by Coomassie blue staining; M: SeeBlue® Pre-stained protein standard. Immunoblot analyses were performed using specific anti-PeAfpA antibody. Parental strain CECT 20796 was loaded as a negative control. Positive PeAfpA producing strains (PDGL11412, PDGL11432 and PDMG11442) are highlighted in red.
C. Evaluation of *afpA* gene copy number in the different PeAfpA-producing strains by qPCR. The Ct signal of *afpA* and L18a was normalized to that of β-*tub*. Results are presented as mean values ± SD of three technical replicates. Under this experimental design, the resulting gene copy number is expected to be 1 for *afpA* in CMP-1, and ≥ 1 for *afpA* in *P. digitatum* transformants.
D. Colony morphology of *P. digitatum* PeAfpA producing strains PDGL11412, PDGL11432 and PDGL11442 compared to the parental strain CECT 20796 after 7 days of growth on PDA and PdMM plates.

PeAfpA processing in the N-terminal region, in which the SP-pro peptide is cleaved out. The yield of recombinant protein after purification reached 4.8 mg l^{-1}, similar to the quantity produced at the same time point by CMP-1 (4.2 mg l^{-1}). No differences in antifungal activity were observed among recombinant and native PeAfpA (Fig. 4C).



(B)

VLYTGQCFKKDNICKYKVNGKQNIAKCPSAANKRCEKDKNKCTFDSYDRKVTCDFRK
VLYTGQCFK
VLYTGQCFKK
NKCTFDSYDR
CTFDSYDR
CTFDSYDRK
VTCDFR
VTCDFRK

Fig. 4. Production and identification of PeAfpA in wild-type *P. expansum* CMP-1 and recombinant *P. chrysogenum* PCMG11552 strains.
A. SDS-PAGE (top) and western blot analysis (bottom) of 10 μl of 5× supernatants of strains grown in *P. chrysogenum* minimal medium (PcMM) for 3, 5, 7 and 10 days. One μg of pure PeAfpA was added as control. SDS-PAGE analysis was visualized by Coomassie blue staining; M: SeeBlue® Pre-stained protein standard. Immunoblot analysis was performed using specific anti-PeAfpA antibody.
B. Peptide mass fingerprinting (PMF) of the recombinant PeAfpA protein purified from PCMG11552 grown in PcMM for 5 days. Peptides obtained by PMF covered 49% of PeAfpA primary sequence (top).
C. Dose–response curve comparing the antifungal activity of native (red circles) and recombinant PeAfpA (blue circles) against *P. digitatum*. Plotted data are mean values ± SD of triplicate samples after 48 h at 25°C.

The feasibility of the expression system to produce PdAfpB depends on the fungal biofactory

In order to expand and compare their applicability, we evaluated the feasibility of both cassettes to produce a second AFP, PdAfpB from *P. digitatum*.

First, PdAfpB production was evaluated in *P. chrysogenum* (Fig. 5A). Several *P. chrysogenum* positive clones were obtained for each transformation event (Fig. S3). SDS-PAGE and western blot analyses of 5-days culture supernatants of positive transformants are depicted in Fig. 5B. No production of recombinant PdAfpB was observed in any of the nine evaluated transformants (Fig. 5B left panel) when using the *afpA* cassette. Conversely, by using the *paf* cassette, PdAfpB production was observed in two of the six culture supernatants evaluated, as confirmed by the immunoreaction observed in western blot analysis (Fig. 5B right panel, in green). These results evidence that, in the conditions tested, we could only achieve production of PdAfpB in *P. chrysogenum* under the control of the *paf* regulatory elements. Analysis of the gene copy number revealed

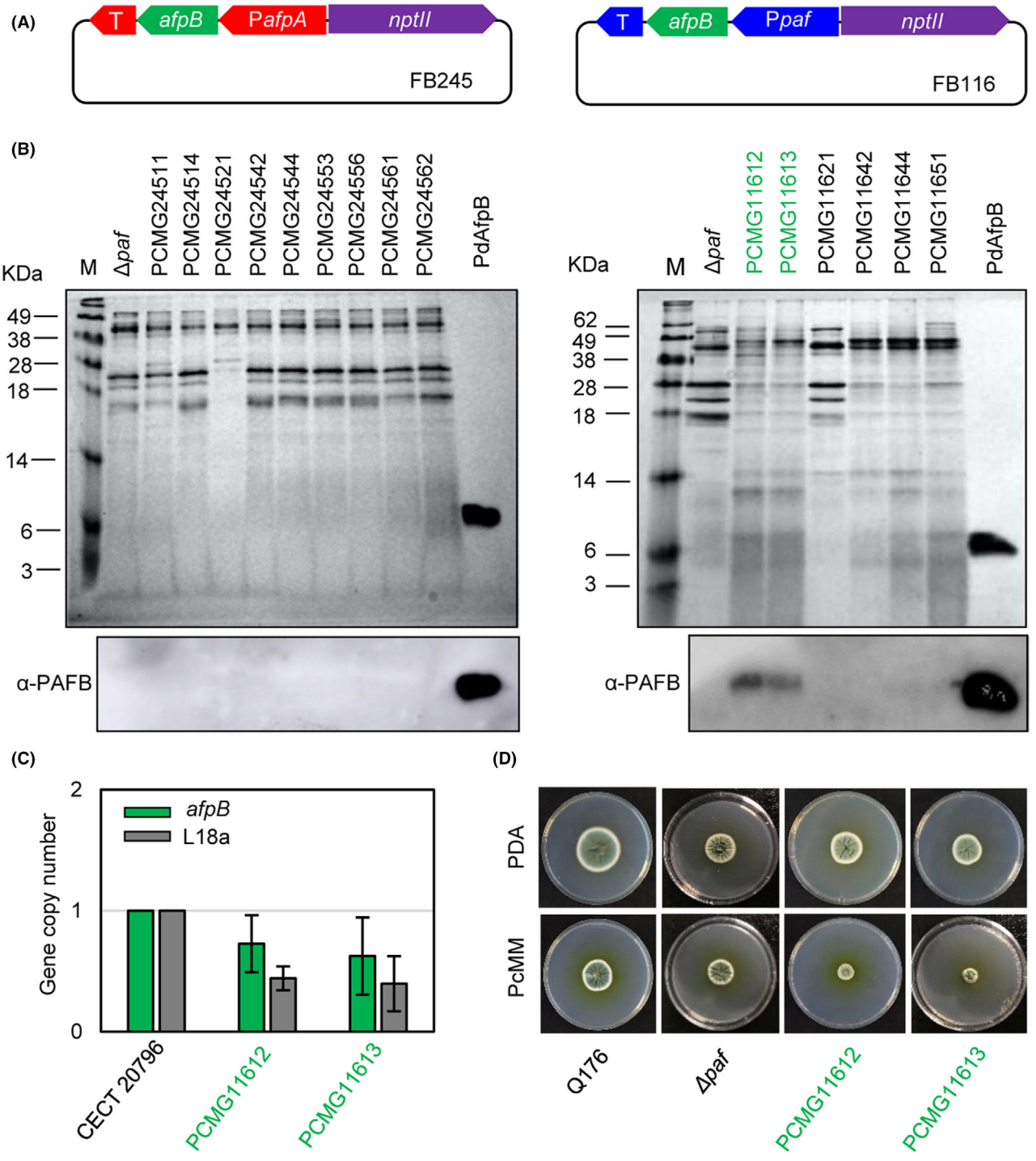


Fig. 5. Analyses of *P. chrysogenum* transformants for PdAfpB production using either the *afpA* or the *paf* cassette.
A. Schematic diagram of the binary vectors FB245 and FB116 used for *P. chrysogenum* transformation.
B. SDS-PAGE (top) and western blot analyses (bottom) of pure PdAfpB (2 μg) and growth supernatants of recombinant strains (10 μl of 10× supernatants loaded per lane) obtained by either FB245 (left) or FB116 (right) transformation. SDS-PAGE analyses were visualized by Coomassie blue staining; M: SeeBlue® Pre-stained protein standard. Immunoblot analyses were performed using specific anti-PAFB antibody. Parental strain Δ*paf* was loaded as a negative control. Positive PdAfpB producing strains (PCMG11612 and PCMG11613) are highlighted in green.
C. Evaluation of *afpB* gene copy number in the different PdAfpB producing strains by qPCR. The Ct signal of *afpB* and L18a was normalized to that of β-*tub*. Results are presented as mean values ± SD of three technical replicates. Under this experimental design, the resulting gene copy number is expected to be 1 for *afpB* in CECT 20796, and ≥ 1 for *afpB* in *P. chrysogenum* transformants.
D. Colony morphology of *P. chrysogenum* PdAfpB producing strains PCMG11612 and PCMG11613 compared to the wild-type Q176 and the parental strain Δ*paf* after 7 days of growth on PDA and PcMM plates.

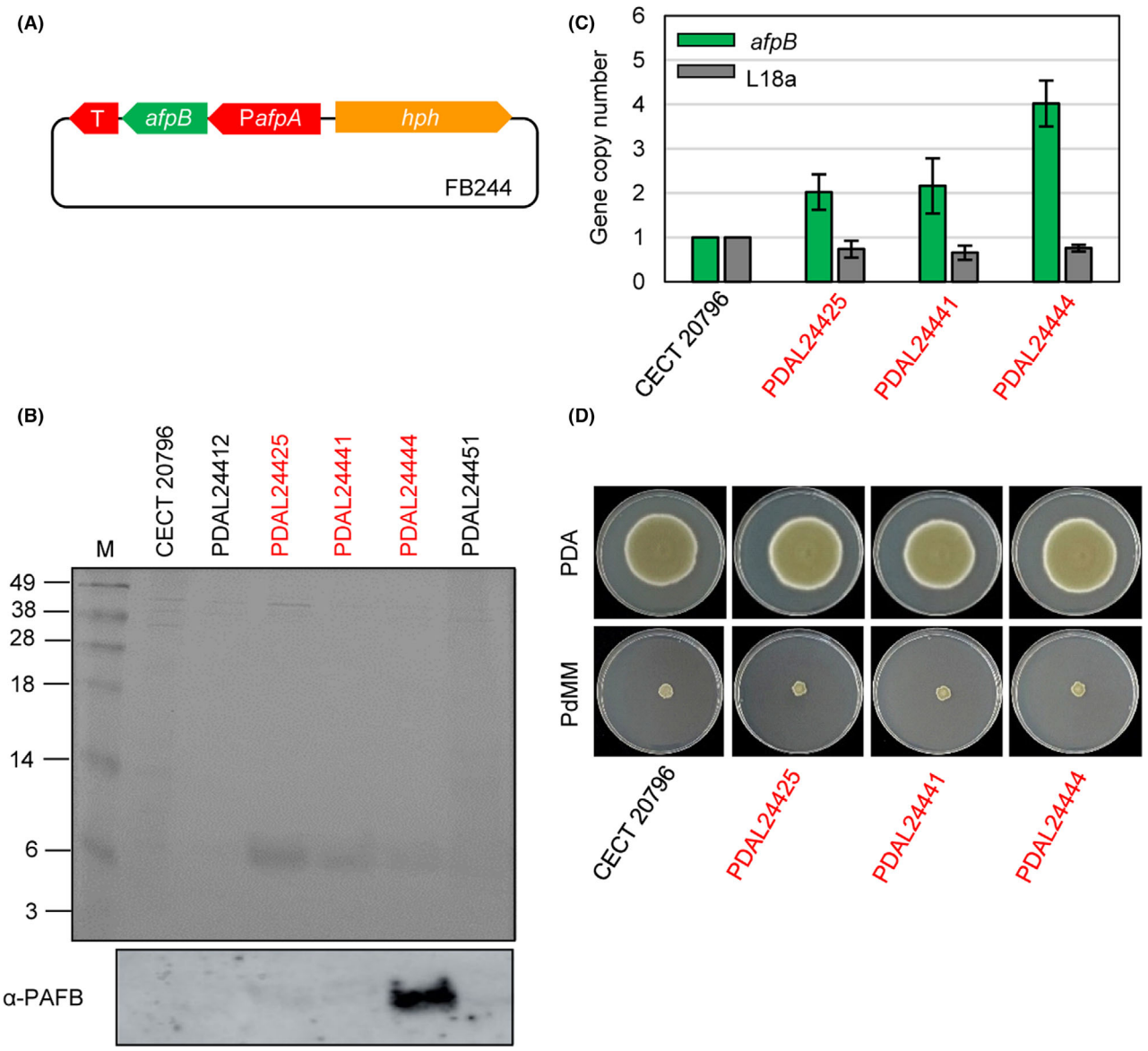


Fig. 6. Analyses of *P. digitatum* transformants for PdAfpB production using the *afpA* cassette.
A. Schematic diagram of the binary vector FB244 used for *P. digitatum* transformation.
B. SDS-PAGE (top) and western blot analyses (bottom) of pure PdAfpB (2 μg) and growth supernatants of recombinant strains (10 μl of 10× supernatants loaded per lane) obtained after transformation with FB244. SDS-PAGE analyses were visualized by Coomassie blue staining; M: SeeBlue® Pre-stained protein standard. Immunoblot analysis was performed using specific anti-PAFB antibody. Parental strain CECT 20796 was loaded as a negative control. Positive PdAfpB producing strains (PDAL24425, PDAL24441 and PDAL24444) are highlighted in red.
C. Evaluation of *afpB* gene copy number in the different PdAfpB producing strains by qPCR. The Ct signal of *afpB* and L18a was normalized to that of β-*tub*. Results are presented as mean values ± SD of three technical replicates. Under this experimental design, the resulting gene copy number is expected to be 1 for *afpB* in CECT 20796, and ≥ 2 for *afpB* in the transformants.
D. Colony morphology of *P. digitatum* PdAfpB producing strains PDAL24425, PDAL24441 and PDAL24444 compared to the parental strain CECT 20796 after 7 days of growth on PDA and PdMM plates.

that only one copy of the P*paf*::*afpB*::T*paf* construction was present in the two *P. chrysogenum* transformants producing recombinant PdAfpB (PCMG11612 and PCMG11613) (Fig. 5C). Growth comparison of both PdAfpB producing clones with that of *P. chrysogenum* Q176 and Δ*paf* strains in solid PDA and PcMM (Fig. 5D) showed no different phenotype on PDA plates, while a reduction of growth in the recombinant strains was observed in PcMM.

Successful PdAfpB production in *P. digitatum* under the control of P*paf* and T*paf* sequences was previously shown (Garrigues *et al.*, 2017; Hernanz-Koers *et al.*, 2018). In this study, PdAfpB production under the control of the *afpA* regulatory elements (Fig. 6A) was evaluated.

SDS-PAGE and western blot analyses of the culture supernatants of five positive *P. digitatum* clones (Fig. S3) are shown in Fig. 6B. A clear PdAfpB-specific signal was only immunodetected in the PDAL24444 supernatant. By contrast, very faint immunoreaction was observed for PDAL24425 and PDAL24441 strains. Gene copy number analyses revealed that PDAL24441 presented three copies of the P*afpA*::*afpB*::T*afpA* construction while the other two transformants presented one copy each (Fig. 6C). As it can be seen in Fig. 6D, none of the three clones showed phenotypic differences to that of the parental strain CECT 20796 in both solid media tested. These results suggest that, in the conditions tested, PdAfpB can also be detected in supernatants of *P. digitatum* transformants obtained under the control of P*afpA* and T*afpA* sequences.

*Comparative analysis of P*afpA *and P*paf *in a luciferase reporter system*

Our results suggest that the effectiveness of P*afpA* and P*paf* depends on the AFP produced and the fungal biofactory. To further compare the strength of both promoter sequences, a reporter system was designed and constructed to drive the expression of the firefly *luciferase* (*luc*) gene. Concurrent expression of the *Nanoluciferase* (*Nanoluc*) gene from the shrimp *Oplophorus gracilirostris* was used as an internal normalization standard.

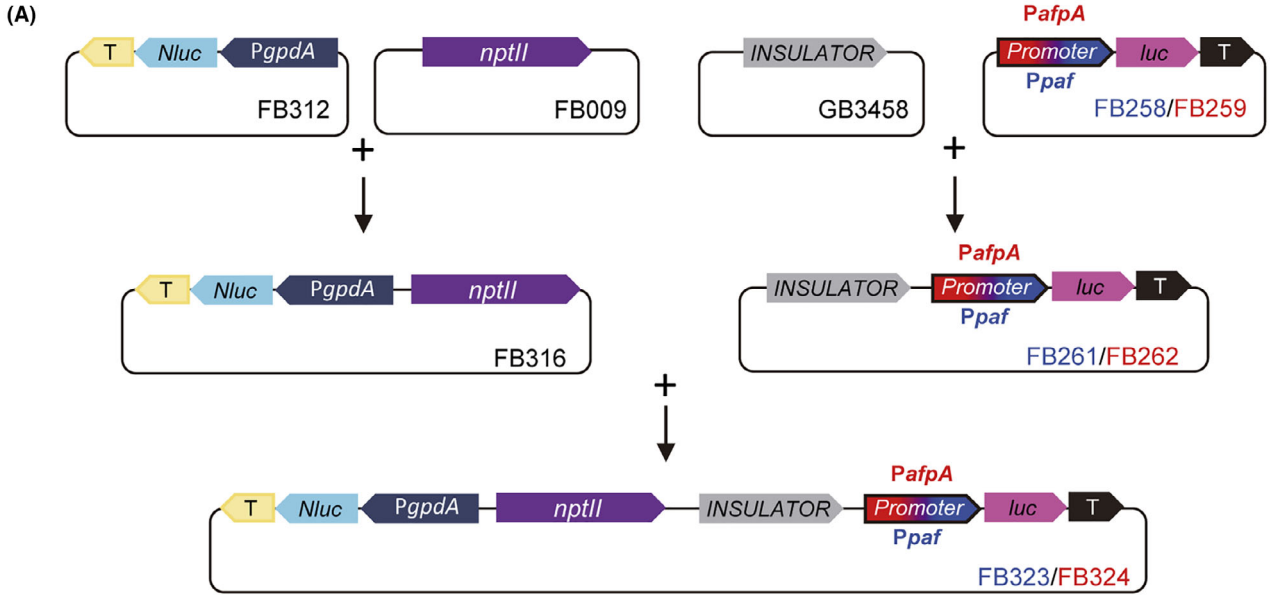
For the development of the FB luciferase reporter system, the CDS from the *Nanoluc* gene was introduced in the FB system by routine protocols (Hernanz-Koers *et al*., 2018; Vázquez-Vilar *et al*., 2020). Genetic elements already adapted to FB (P*gpdA*, T*tub* and the CDS from the *luc* gene) were also used (Table 1). Next, single TUs combining either P*paf* or P*afpA* with the *luc* CDS were assembled. In parallel, the TU for *Nanoluc* expression was obtained and combined with the geneticin-resistant marker (FB009) to generate the binary vector FB316. Finally, vectors FB323 and FB324 were generated and used for fungal transformation (Table 1 and Fig. 7A).

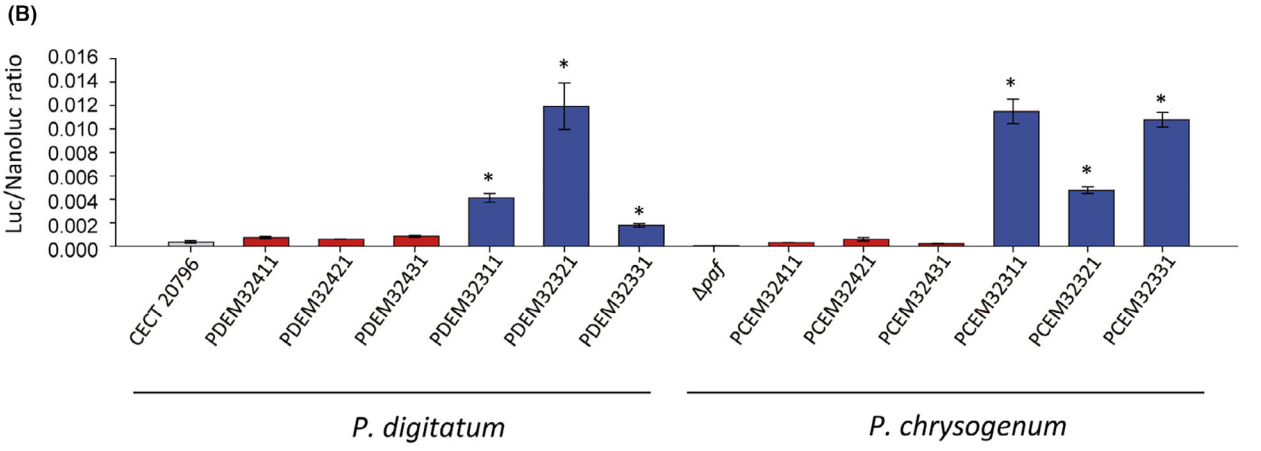
First, three independent positive clones for *luc* expression under the control of either P*afpA* or P*paf* in both *P. digitatum* and *P. chrysogenum* (Fig. S4) were analysed in 1 ml of PdMM or PcMM after 2 days of growth. Under these growing conditions, very faint *luc* expression under the control of P*afpA* was recorded in all the transformants analysed (Fig. 7B, in red). Although slightly higher in the transformants, *luc* expression was not statistically significant to that showed by the control strains. On the other hand, under the control of P*paf*, *luc* expression was significantly higher than that observed with P*afpA* and in the control strains, regardless of the fungal factory (Fig. 7B, in blue). Subsequently, one independent clone for each condition (PDEM32421, PDEM32311, PCEM32431 and PCEM32331) was chosen to monitor gene expression during time-course experiments in higher volumes of MM, mimicking the conditions used for AFP production (Fig. 7C). Promoter strength was analysed after 2, 5 and 10 days of growth. Regardless of the fungal biofactory and the promoter used, the highest *luc* expression was achieved at day 5. In *P. digitatum*, the strength of P*afpA* was significantly higher than that of P*paf* to drive *luc* expression at days 2 and 5, whereas at day 10 no significant differences in *luc* expression between both promoters were observed. By contrast, in *P. chrysogenum*, the P*paf* was stronger than the P*afpA* at days 2 and 5, and no significant differential expression was detected at day 10. Results obtained indicated higher *luc* expression, and thus higher promoter activation, under the growing conditions mimicking those employed for AFP production.

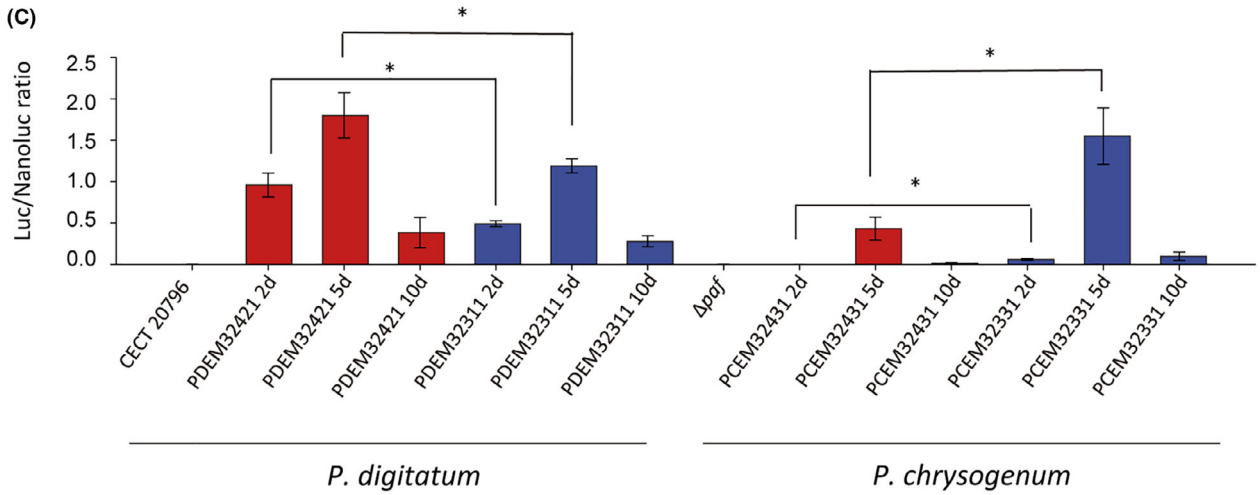
Discussion

In this study, we explored the feasibility of a new *P. expansum*-based *expression system adapted to the modular cloning platform FB for AFP production in two Penicillium* species, the biotechnologically relevant *P. chrysogenum* (Jami *et al*., 2010), and the non-mycotoxigenic postharvest pathogen of citrus fruit *P. digitatum* (Marcet-Houben *et al*., 2012), which has been already demonstrated to produce some AFPs in high yields (Sonderegger *et al*., 2016; Garrigues *et al*., 2017). Combinatorial experiments exchanging the different FB DNA parts allowed accurate comparison of the new *afpA* cassette with that of the previously developed *paf*

Fig. 7. Luciferase assay for testing P*afpA* and P*paf* strength in *Penicillium*.
A. Schematic diagram of TU assemblies to drive the expression of the *luciferase* (*luc*) gene under the control of P*afpA* or P*paf* (FB258 and FB259) and the *Nanoluciferase* (*Nanoluc*) gene under the control of P*gpdA* (FB312). Final vectors obtained with geneticin (*nptII)* resistant marker (FB009) were used for transformation of *P. chrysogenum* and *P. digitatum* (FB323 and FB324). An insulator sequence (GB3458) was used to allow the binary assembly of the plasmids containing the *luc* TU with the plasmid containing *Nanoluc* TU and geneticin resistant marker.
B. Luciferase/Nanoluciferase signal ratio of 3 independent transformants for each construct in *P. digitatum* and *P. chrysogenum* at 2 days of growth in minimal medium (PdMM or PcMM, respectively). Values are represented as the mean ± standard error (SE). Asterisks (*) denote statistically significant differences in comparison to control values (ANOVA and Tukey's HSD test, $P < 0.05$).
C. Luciferase/Nanoluciferase signal ratio of a selected transformant for each construct in *P. digitatum* and *P. chrysogenum* at 2, 5 and 10 days of growth in PdMM or PcMM, respectively. Values are the means ± SE from three independent replicates. Asterisks (*) denote statistically significant differences between promoters at each time-point ($P < 0.05$, Student's *t*-test).







cassette for the production of PeAfpA and PdAfpB. As evidenced by our results, the performance of both promoters depends on the AFP sequence, the growing conditions and the biofactory.

In this work, PeAfpA, the most effective AFP from *P. expansum* (Garrigues *et al*., 2018), was only produced with the *afpA* cassette and resulted in higher yields in *P. chrysogenum* than in *P. digitatum*. PeAfpA shows a strong antifungal potency against human and plant pathogens, lack of cytotoxicity and significant *in vivo* protection against phytopathogenic fungi (Garrigues *et al*., 2018; Gandia *et al*., 2020). Thus, PeAfpA can be considered a promising compound for application in agriculture, but also in medicine or food preservation (Garrigues *et al*., 2018; Martínez-Culebras *et al*., 2021). Natural production of PeAfpA is achieved from wild-type *P. expansum* CMP-1 strain, reaching yields higher than 100 mg l^{-1} (Garrigues *et al*., 2018). Despite the high production of PeAfpA, *P. expansum* is a phytopathogenic fungus of concern due to the production of mycotoxins patulin and citrinin (Tannous *et al*., 2018), and thus the possibility of an alternative and safer cell factory for PeAfpA production is of relevance. Here, we have demonstrated that *P. chrysogenum* is an efficient cell factory for PeAfpA production using the new *afpA* cassette. Although the level of recombinant PeAfpA at day 5 is similar to that produced by *P. expansum* wild-type strain at the same time point (4.8 vs. 4.2 mg l^{-1}, respectively), it is far from the maximum level produced by CMP-1 after 10 days of growth (Garrigues *et al*., 2018). Further research to improve recombinant PeAfpA yields is in progress. In this work, we have used the *P. chrysogenum* Δ*paf* mutant as recipient strain to avoid co-expression of the wild-type PAF. However, the use of *P. chrysogenum* wild-type strain as factory is also feasible provided that specific AFP purification protocols are employed, as already described for PAFB purification (Huber *et al*., 2019).

On the other hand, the production of PdAfpB has also been evaluated in this work. PdAfpB is the only AFP encoded in the fungus *P. digitatum*, although it is not naturally produced (Garrigues *et al*., 2016). Its biotechnological production was successfully achieved in *P. digitatum* with the use of the *paf* cassette (Garrigues *et al*., 2017). PdAfpB has shown high antifungal activity against mycotoxin-producing fungi (Martínez-Culebras *et al*., 2021) and protection against *Botrytis cinerea* infection in tomato leaves and plants (Garrigues *et al*., 2018; Shi *et al*., 2019). In this work, we have demonstrated that under the regulation of the P*paf* sequence, *P. chrysogenum* recombinant strains do not reach the levels of PdAfpB produced by *P. digitatum* transformants (12-20 mg l^{-1}) (Garrigues *et al*., 2017). Finally, the *afpA* cassette did not result in PdAfpB production in *P. chrysogenum*, while in *P. digitatum* transformants faint levels of PdAfpB were achieved. Thus, in the conditions tested, PdAfpB was only detected in supernatants of *P. digitatum* transformants, being the P*paf* sequence the most appropriate promoter for PdAfpB production.

In this study, we confirm that *P. chrysogenum* and *P. digitatum* can be good biofactories for AFP production, although the election of both the promoter and the fungus is dependent on the specific AFP. The production of these small CRPs in bacterial systems often fails due to incorrect folding and disulphide bridge formation (Kiedzierska *et al*., 2008; Rosano and Ceccarelli, 2014). Transient production in plants is also an attractive alternative (Shi *et al*., 2019), although fungal systems in general, and filamentous fungi in particular, stand out for better yields and single-step purification procedures (López-García *et al*., 2010; Virágh *et al*., 2014; Sonderegger *et al*., 2016; Garrigues *et al*., 2017; Tóth *et al*., 2018). Another CRP group of interest comprises antifungal plant defensins. Their heterologous production has been achieved in both *E. coli* (Bleackley *et al*., 2016) and *P. pastoris* (Hayes *et al*., 2013). The evaluation of both the *afpA*- and *paf*-cassette for the production of these CRPs in filamentous fungi will be considered in the near future.

Efficient systems for homologous and heterologous gene expression in *P. chrysogenum* (Graessle *et al*., 1997; Díez *et al*., 1999; Zadra *et al*., 2000), as well as new promoters for strain engineering have been described (Polli *et al*., 2016), and this fungus has been extensively used in biotechnology as cell factory for the production of biomolecules (Jami *et al*., 2010). For AFP production in *P. chrysogenum*, the *paf* cassette turned out to be a perfect tool for the generation of correctly folded and active proteins with purification yields in the range from 3 (*N. fischeri* NFAP) to 80 mg l^{-1} (*P. chrysogenum* PAF) (Sonderegger *et al*., 2016). Here, the *paf* cassette was not successful for PeAfpA production and only minor production of PdAfpB was achieved. The original *paf* cassette included the *paf* SP-pro sequence to warrant the secretion of AFPs, as described for the above-mentioned NFAP and PAF (Sonderegger *et al*., 2016), but also for *P. chrysogenum* PAFB and PAFC (Huber *et al*., 2018; Holzknecht *et al*., 2020) and *P. expansum* PeAfpB and PeAfpC (Garrigues *et al*., 2018). In this work, the production of both PeAfpA and PdAfpB was evaluated using either the *afpA* or the *paf* cassette containing the corresponding native SP-pro sequence, suggesting that for AFP production in *P. chrysogenum* the *paf* SP-pro sequence might be more appropriate. However, in a previous work, the production of PeAfpA in *P. chrysogenum* with the *paf* regulatory elements and the *paf* SP-pro sequence failed (Garrigues *et al*., 2018), indicating that the impossibility to detect PeAfpA in

culture supernatants is not due to either the native *afpA* or *paf* SP-pro sequence, but to the regulatory sequences which would to some extent compromise the stability of the resulting mRNA.

With respect to *P. digitatum* as AFP biofactory, the successful production of PdAfpB under the control of P*paf* and T*paf* sequences (Garrigues *et al*., 2017) prompted us to evaluate PdAfpB production under the control of P*afpA* and T*afpA*. However, the protein was produced in very low levels compared to that obtained with the *paf* cassette (Garrigues *et al*., 2017). It should be noted that its native SP-pro sequence allowed production, secretion and optimal purification of the protein with similar yield to that achieved with the *paf* SP-pro sequence (Garrigues *et al*., 2017), pointing out the importance of the regulatory elements also observed in *P. chrysogenum*. Interestingly, minor amounts of PeAfpA were produced with the *afpA* regulatory elements, whereas no protein was detected under the control of P*paf* and T*paf*, suggesting a role of the AFP amino acid sequence in protein production/accumulation.

Special attention was paid to the correct processing of recombinant PeAfpA produced by *P. chrysogenum*. In our previous work, we demonstrated that only one amino acid difference (Leu1) in PdAfpB determined the correct folding/unfolding capabilities of the protein after denaturation procedures, although its antifungal activity was not affected (Garrigues *et al*., 2017). Here we show that *P. chrysogenum* proteases efficiently recognized the *P. expansum afpA* SP-pro sequence during protein maturation, since the protein is secreted and the N-terminal region of recombinant PeAfpA is equal to that of the wild-type protein (Garrigues *et al*., 2018).

The dependence of the promoter efficiency on each AFP has also been observed for the constitutive P*gpdA* sequence from *Aspergillus nidulans*. Attempts to detect PdAfpB in *P. digitatum* transformants under P*gpdA* failed, and transformants showed a drastic reduction of axenic growth, abnormal hyphal morphology and delayed conidiogenesis (Garrigues *et al*., 2016). By contrast, P*gpdA* was successful to produce the antifungal protein NFAP from *N. fischeri* in *A. nidulans*, although in low levels (1.7 mg l^{-1}), while negatively affected hyphal growth and germination of transformed strains (Galgóczy *et al*., 2013). Recently, the side-by-side comparison of *paf*, *pafB* and *xylP* promoters to produce PAFB in *P. chrysogenum* revealed that the *paf* promoter efficiency was similar to that of the *pafB*, but superior to that of the *xylP* promoter (Huber *et al*., 2019).

Our results highlight the difficulties associated with producing antimicrobial peptides and proteins in fungal factories. The toxicity of AFPs against the host strain might partially explain the differences in production. AFPs tested in this work show antifungal effect towards both *P. chrysogenum* and *P. digitatum*. Remarkably, only one of the three *P. chrysogenum* strains producing recombinant PeAfpA (PCMG11532) had conidia production affected, although this could be also related to gene copy number (3 copies for PCMG11532 *vs* 2 copies for PCMG11522 and PCMG11552). Moreover, we have not found any relationship between gene copy number and protein overproduction. The clone PCMG11552 selected as the highest recombinant PeAfpA producer contains two copies of the P*afpA*::*afpA*::T*afpA* construction, the same number of copies than the clone PCMG11522 in which PeAfpA production is lower, suggesting the effect of the integration loci. In addition, the integration of three copies of the gene does not improve PeAfpA production (clone PCMG11552 *vs* PCMG11532). In *P. digitatum*, the integration of one copy of the P*afpA*::*afpA*::T*afpA* did not result in overproduction of the protein, and detection was only accomplished by western blot. Whether production of recombinant PeAfpA in *P. digitatum* needs at least two copies of the *afpA* gene as observed in *P. chrysogenum* requires further studies. Regarding transformants for PdAfpB overproduction, only one copy of the P*paf*::*afpB*::T*paf* construction was present in the two *P. chrysogenum* transformants (PCMG11612 and PCMG11613) producing faint quantities of recombinant PdAfpB while in *P. digitatum*, the strain producing the highest quantities of protein (PDAL24444) presented three copies of the P*afpA*::*afpB*::T*afpA* construction. All together, these results again indicate the importance of the regulatory sequences, the AFP coding sequence and the fungal biofactory.

Finally, we have constructed a reporter expression system for promoter strength analysis in *P. chrysogenum* and *P. digitatum*. The luciferase reporter assay is commonly used as a tool to study gene expression at the transcriptional level since it gives quantitative measurements instantaneously. In the system, the *luc* gene was placed under the control of either P*afpA* or P*paf* sequences. For comparison and internal calibration, the gene encoding the *Nanoluc* under the control of the P*gpdA* was also integrated into the same expression cassette. Although the use of the ratio luc/nanoluc eliminates potential interferences by variations in growth, gene copy number or genome position of the transferred DNA, differences among biological replicates were observed, as described for other reporter systems with internal calibration (Polli *et al*., 2016). Our data clearly showed differences in gene expression depending on growing conditions. When fungi were grown in low medium volumes with reduced aeration, *luc* expression was overall much lower. However, in these conditions the performance of P*paf* was much better than that of P*afpA*. In the growing conditions used for AFP production, both promoters showed a similar expression trend,

and the highest expression was achieved at day 5. Intriguing is the lack of correlation between the strength of promoters in the reporter system and that found during protein production experiments. In *P. chrysogenum*, the strength of P*paf* was higher than that of P*afpA*, but PeAfpA production was only achieved under the control of its own regulatory sequences while minor quantities of PdAfpB were achieved under the control of P*paf* and T*paf*. These results strengthen the importance of the target AFP to be produced. In *P. digitatum*, in which the performance of P*afpA* was slightly higher than that of P*paf*, minor quantities of PeAfpA were detected only in transformants containing the P*afpA*::*afpA*::T*afpA* construction. As reported earlier, high yields of PdAfpB were obtained under the control of P*paf* and T*paf* (Garrigues *et al*., 2017; Hernanz-Koers *et al*., 2018) whereas very faint production was achieved in transformants containing the P*afpA*::*afpB*::T*afpA* construction.

In summary, this study describes the performance of a new *afp* promoter, P*afpA*, for the production of AFPs in two fungal biofactories, in comparison with the well-known P*paf*. In the conditions tested, our findings suggest that there is neither a universal *afp* promoter nor universal fungal biofactory for the production of a given AFP. The reporter expression system for promoter strength analysis in *P. chrysogenum* and *P. digitatum* developed here strengthen the importance of the target AFP to be produced. Future efforts are directed to analyze new fungal promoters and to clarify the role of the *afp* regulatory and SP-pro sequences to optimize protein production.

Experimental procedures

Microorganisms, media and culture conditions

Fungal strains used in this study were *P. digitatum* CECT 20796 (PHI26) (Marcet-Houben *et al*., 2012), *P. chrysogenum* wild-type strain Q176 and *P. chrysogenum* Δ*paf* (Hegedüs *et al*., 2011). *P. digitatum* and *P. chrysogenum* Δ*paf* were used as parental strains for fungal transformation. Fungi were cultured on PDA (Difco-BD Diagnostics, Sparks, MD, USA) plates for 7–10 days at 25°C. For transformation, vectors generated were amplified in *E. coli* JM109 grown in Luria Bertani (LB) medium supplemented with either 25 μg ml^{-1} chloramphenicol, 50 μg ml^{-1} kanamycin, 100 μg ml^{-1} spectinomycin or 100 μg ml^{-1} ampicillin at 37°C depending on the vector. *Agrobacterium tumefaciens* AGL-1 strain was cultured in LB medium with 20 μg ml^{-1} rifampicin at 28°C.

To compare the growth of the *P. chrysogenum* and *P. digitatum* transformants with that of parental strains on solid media, 5 μl of conidial suspension (5×10^4 conidia ml^{-1}) were deposited on the center of PDA and PcMM or PdMM plates (Sonderegger *et al*., 2016). Colony morphology was assessed and compared by visual inspection after 6–7 days of growth.

Design, domestication and assembly of genetic elements to generate different expression and AFP production vectors through FB system

All the genetic elements previously available or generated in this study are described in Table 1. GB0096 element codes for the firefly *luciferase* (*luc*) gene (https://gbcloning.upv.es/feature/GB0096/), whereas GB3458 element encodes an insulator (*AtS/MAR10*) from *Arabidopsis thaliana* (Pérez-González and Caro, 2019), both obtained as GB plasmids. The regulatory elements P*afpA* and T*afpA*, and the CDS for the *P. expansum afpA* gene (PEX2_042150) were designed according to GB rules (https://gbcloning.upv.es) and provided by an external company (IDT, Integrated DNA Technologies) as synthetic genes (gBlocks™ gene fragments). The CDS for *Nanoluciferase* (*Nanoluc*, from the shrimp *Oplophorus gracilirostris*) was codon-optimized for fungal expression and ordered as a synthetic gene. Each single genetic element was ligated into the domestication entry vector pUPD2 through protocols described previously (Hernanz-Koers *et al*., 2018; Vazquez-Vilar *et al*., 2020) to obtain FB107, FB108, FB109 and FB310 elements (Table 1). Positive *E. coli* clones were confirmed by routine PCR amplifications using external specific primers designed for pUPD2 vectors (Hernanz-Koers *et al*., 2018) (Table S1) and confirmed by Sanger sequencing.

Multipartite assemblies to obtain the TUs to drive the production of PeAfpA and PdAfpB with the *afpA* or *paf* cassettes (Table 1) were carried out as described previously (Hernanz-Koers *et al*., 2018; Vázquez-Vilar *et al*., 2020). The TU to produce PdAfpB under the control of P*paf* and T*paf* (Table 1) was generated in a previous work (Hernanz-Koers *et al*., 2018). To compare the efficiency of the promoters P*paf* and P*afpA* to drive the expression of the *luc* gene, the elements FB258 and FB259 were obtained. The TU unit to drive the expression of the *Nanoluc* gene under the control of the P*gpdA* promoter (FB007) and T*tub* terminator (FB002) was also obtained (FB312; Table 1).

Binary assemblies (FB114, FB115, FB158, FB159, FB116, FB244 and FB245) (Table 1) were obtained as previously described (Hernanz-Koers *et al*., 2018; Vázquez-Vilar *et al*., 2020) and combine the TUs for the production of PeAfpA (FB112 and FB146) and PdAfpB (FB230 and FB033) with the TUs used as fungal positive selection markers (hygromycin (*hph*) for *P. digitatum* and geneticin (*nptII*) for *P. chrysogenum*, which correspond to FB003 and FB009 elements, respectively) (Fig. 1). Binary assembly FB036 that combines FB033 and FB003 was previously obtained (Hernanz-Koers *et al*.,

2018). FB316, FB323 and FB324 were obtained using binary assembly to combine the *Nanoluc* TU with FB009 element and with *insulator* TU.

Positive *E. coli* clones and correct assembly were confirmed by restriction and/or PCR analyses using combinations of the universal specific primers designed for pDGB3 vectors (Hernanz-Koers *et al*., 2018) (Table S1). All binary vectors generated that would later be used for fungal transformation were introduced into *A. tumefaciens* AGL-1 strain by electroporation. These binary vectors corresponded to FB115, FB159, FB245 and FB116 for *P. chrysogenum* transformation; and FB114, FB158 and FB244 for *P. digitatum* transformation (Table 1).

Fungal transformation

Fungal transformation of *P. digitatum* CECT 20796 and *P. chrysogenum* Δ*paf* with the corresponding FB binary vectors described before (Table 1) was performed following the *A. tumefaciens*-mediated transformation (ATMT) protocol previously described (Harries *et al*., 2015; Vázquez-Vilar *et al*., 2020). The *A. tumefaciens* AGL-1 strain was used for fungal transformation (Gandía *et al*., 2014). *P. digitatum* and *P. chrysogenum* transformants were selected in 25 μg ml^{-1} hygromycin B or 25 μg ml^{-1} geneticin (G418) (Invivogen, San Diego, CA, USA), respectively. All transformants were confirmed by PCR (Table S1; Fig. S1, S3 and S4) using genomic DNA isolated as described previously (Khang *et al*., 2006), and subsequently by 1% agarose gel electrophoresis.

Dual luciferase assays

Penicillium chrysogenum and *P. digitatum* transformants were grown in triplicate in 2 ml tubes containing 1 ml or in 100 ml flasks containing 25 ml of either PcMM or PdMM for 2, 5 or 10 days at 25°C and 150 rpm. Mycelia were then collected by centrifugation (12 000 *g*, 10 min at 4°C) and immediately frozen in liquid nitrogen. The frozen mycelia (~ 20 mg) were homogenized in 180 μl of Passive Lysis Buffer (Promega, Madison, WI, USA) with a pestle. Luciferase assay was performed with the Dual-Glo® Luciferase Assay System (Promega), following the manufacturer's protocol with minor modifications. Briefly, 10 μl of the fungal homogenized extract were transferred to a white 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA) and mixed with 40 μl of Luciferase Reagent to measure Luciferase luminescence. Thereafter, 40 μl of Stop&Glow Reagent were added and Nanoluciferase luminescence signal quantified. Luciferase and Nanoluciferase luminescence signals were determined using a CLARIOStar multimode microplate reader (BMG LABTECH GmbH, Quakenbrück, Germany) with a measurement of 10 s and a delay of 2 s. Measurements were repeated three times.

Protein production and western blot analyses

For protein production, 25 ml of PcMM or PdMM were inoculated with a final concentration of 10^6 conidia ml^{-1} of transformants and were incubated at 25°C and 150 rpm for 5 (*P. chrysogenum*) or 11 days (*P. digitatum*). Total proteins from supernatants and purified PeAfpA and PdAfpB were separated by SDS-PAGE (16% polyacrylamide gels) and transferred to Amersham Protran 0.20 μm NC nitrocellulose transfer membrane (GE Healthcare Life Sciences, Chicago, IL, USA) as described (Garrigues *et al*., 2018). Protein detection was accomplished using anti-PeAfpA antibody diluted 1:2,500 (Garrigues *et al*., 2018) or anti-PAFB antibody diluted 1:1,000 (Garrigues *et al*., 2017). As secondary antibody, 1 : 20 000 dilution of ECL NA934 horseradish peroxidase donkey anti-rabbit (GE Healthcare Life Sciences) was used and chemiluminescent detection was performed with ECL™ Select Western blotting detection reagent (GE Healthcare Life Sciences) using Amersham Imager 680 (GE Healthcare Life Sciences). The experiments were repeated at least twice.

PeAfpA purification and peptide mass fingerprinting (PMF)

PeAfpA purification from 5-day PcMM culture supernatants of *P. expansum* CMP-1 and *P. chrysogenum* PCMG1152 was accomplished by cation exchange chromatography following previously published procedures (Garrigues *et al*., 2018). Peptide mass fingerprinting (PMF) was performed in the proteomics facility of SCSIE University of Valencia (Spain) as previously described (Garrigues *et al*., 2018).

Determination of afpA *and* afpB *gene copy number*

Evaluation of *afpA* and *afpB* gene copy number in the different *P. digitatum* or *P. chrysogenum* transformants was performed as previously described (Garrigues *et al*., 2016) with some modifications. Briefly, qPCR was applied to genomic DNA of the *P. digitatum* or *P. chrysogenum* AFP-producing strains with primers specific for *afpB* (OJM466/OJM467), *afpA* (OJM588/OJM589), and for the single copy control genes β-*tubulin* (β-*tub*) (OJM85/OJM86) and the 60S ribosomal protein-encoding gene L18a (OJM151/OJM152) (Table S1). The Ct signal of *afpB*, *afpA*, and L18a was normalized to that of β-*tub* used as internal control and to the signal of the parental *P. digitatum* CECT 20796 (for *afpB)*, or *P. expansum*

CMP-1 (for *afpA)*. Results are presented as mean values ± SD of three technical replicates.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Molecular characterization of *P. chrysogenum* and *P. digitatum* transformants for PeAfpA production. (A) Schematic localization of different primers used for PCR analyses. (B) PCR amplification of genomic DNA of the distinct *P. chrysogenum* strains with different primer pairs as indicated. Positive transformation strains (in red) showed expected amplicons either with primers OJM620/OJM621 (1.8 kb) or OJM483/484 (2 kb). (C) PCR amplification of genomic DNA of the distinct *P. digitatum* strains with different primer pairs as indicated. Positive transformants (in red) showed expected amplicons either with primers OJM620/OJM621 (1.8 kb) and OJM483/484 (2 kb). Genomic DNA from *P. digitatum* CECT 20796 and *P. chrysogenum* Δ*paf* strains, FB vectors FB003, FB009 and distilled H_2O were used as negative controls. FB vectors FB115 and FB159 were used as positive controls.

Fig. S2. Conidia production on PDA plates of *P. chrysogenum* PeAfpA producing strains. Conidia production was determined as previously described (Gandía *et al*., 2014). Data show the mean ± standard deviation (SD) of three replicates of the conidia/cm^2 produced per plate. Statistical analyses were conducted using the Excel statistical package. Asterisk shows significant differences with the parental strain Δ*paf* (t-student test, $P < 0.05$).

Fig. S3. Molecular characterization of different *P. chrysogenum* and *P. digitatum* transformants for PdAfpB production. (A) Schematic localization of different primers used for PCR analyses. Genetic constructions are drawn to scale. (B) PCR amplification of genomic DNA of the *P. chrysogenum* strains with different primer pairs as indicated. Positive transformation strains (in red) showed expected amplicons either with primers OJM620/OJM621 (1.8 kb) and OJM483/484 (2 kb). (C) PCR amplification of genomic DNA of the *P. digitatum* strains with OJM466/OJM621 primer pair. Positive transformation strains (in red) showed the expected amplicon (1 kb). Genomic DNA from *P. chrysogenum* Δ*paf* and *P. digitatum* CECT 20796 strains, FB vector FB003 and distilled H_2O were used as negative controls. FB vectors FB245 and FB116 were used as positive controls.

Fig. S4. Molecular characterization of *P. chrysogenum* and *P. digitatum* transformants for luciferase reporter system. (A) Schematic localization of different primers used for PCR analyses. (B) PCR amplification of genomic DNA of the distinct *P. chrysogenum* strains with different primer pairs as indicated. Positive transformation strains (in red) showed expected amplicons either with primers OJM509/OJM555 (1.5 kb), OJM620/522 (2.8 kb) or OJM501/522 (2 kb). (C) PCR amplification of genomic DNA of the distinct *P. digitatum* strains with different primer pairs as indicated. Positive transformants (in red) showed expected amplicons either with primers OJM509/OJM555 (1.5 kb), OJM620/522 (2.8 kb) or OJM501/522 (2 kb). Genomic DNA from *P. digitatum* CECT 20796 and *P. chrysogenum* Δ*paf* strains, FB vector FB009 and distilled H_2O were used as negative controls. FB vectors FB323 and FB324 were used as positive controls.

Table S1. Primers used in this study for the molecular characterization of different transformants and for qPCR analyses.

