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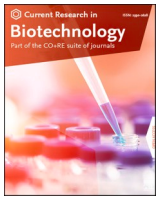

Agrobacterium tumefaciens-mediated transformation for the genetic modification of the biotechnologically relevant fungus *Aspergillus vadensis* through synthetic biology

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ABSTRACT

In the last years, many research efforts have been applied for the development of filamentous fungi as hosts for heterologous protein production. *Aspergillus vadensis* CBS 113365, a close relative of the industrial workhorse *Aspergillus niger*, has been suggested as a more suitable cell factory as it does not acidify the culture medium and produces very low levels of secreted proteases. Therefore, efficient methods and tools that allow the genetic manipulation and exploitation of this biotechnologically relevant fungus are needed. To date, only protoplast-mediated transformation and classical cloning strategies have been implemented for *A. vadensis* genetic modification, which decreases the exploitation capacity of this fungus at the industrial level. In this study, we have adapted and implemented an *Agrobacterium tumefaciens*-mediated transformation protocol for *A. vadensis* for the first time, and applied the FungalBraid system to genetically modify this species by means of synthetic biology. As proof of concept, we have successfully complemented and fluorescently labelled a uridine auxotrophic *A. vadensis pyrA*⁻ strain and generated *A. vadensis* mutants carrying the *Penicillium expansum*-based expression cassette for the heterologous production of the antifungal protein PeAfpA from *P. expansum*. Even though we have yet to find the conditions that trigger PeAfpA production in this species, the implementation of the ATMT method reported here, along with the application of the FungalBraid system, will greatly aid in this task and will facilitate the exploitation of *A. vadensis* as a fungal workhorse for protein production for multiple biotechnological applications.

1. Introduction

In recent decades, the genus *Aspergillus* has become one of the most widely studied and exploited groups of filamentous Ascomycete fungi due to their application as hosts for the biotechnological production of metabolites and recombinant proteins (Meyer et al., 2011). In fact, fungal enzymes, mainly from *Aspergillus,* currently make up more than half of the enzymes used in the industry (Salazar-Cerezo et al., 2023), reflecting the relevance of these species as cell factories. To date, much of this research has been conducted on the well-established fungal platforms *Aspergillus niger* and *Aspergillus oryzae*, which are famous for the production of organic acids and for their high protein secretion abilities (Punt et al., 2002; Cairns et al., 2021). However, while great advances have been made in the (over-)production of heterologous proteins, yields have not yet reached the levels obtained for homologous proteins (Culleton et al., 2013), predominantly due to high protease secretion and media acidification, which would promote protein degradation. In this sense, *Aspergillus vadensis* CBS 113365 and strains thereof have been suggested as a potential alternative for heterologous protein production at the industrial level, as they produce almost no extracellular proteases and do not acidify the culture media (de Vries et al., 2004). Therefore, efficient methods and tools that allow the genetic manipulation and exploitation of this biotechnologically relevant fungal strain are required.

So far, different genetic transformation methods have been established for *Aspergillus* species. These include mainly the protoplast-mediated transformation (PMT), *Agrobacterium tumefaciens*-mediated transformation (ATMT) and electroporation, and other less exploited methods such as the biolistic or shock-wave-mediated transformation methods (Li et al., 2017; Garrigues et al., 2021). By far, PMT has become

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the most commonly applied transformation method in Aspergilli. Nevertheless, ATMT is considered a more advantageous method than PMT for many reasons. In ATMT, fungal spores (and other structures e.g., hyphae or fruiting bodies) can be used directly for transformation instead of the osmotically fragile protoplasts, in which cell wall deconstruction depends on the availability of commercial enzyme cocktails that are beginning in many cases to be discontinued (Casado-del Castillo et al., 2021). In addition, contrary to PMT, ATMT avoids the incorporation of vector DNA into the genome, exclusively introducing the desired DNA sequence together with the selectable marker (Li et al., 2017). Moreover, ATMT has opened the field of molecular genetics for fungi that were difficult to transform via PMT or for which the traditional protocols failed to yield stable DNA integration (Michielse et al., 2005).

In the recent years, Synthetic Biology (SynBio) has revolutionized genetic and metabolic engineering of a great variety of organisms (Serrano, 2007), including filamentous fungi (Mózsik et al., 2022), by providing new methods and tools for the generation of standard, ready-to-use, modular genetic elements. These high-throughput modular cloning methodologies clearly represent a breakthrough over classical cloning, as type IIS restriction enzymes make these new cloning alternatives fully reusable by allowing iterative cloning strategies. In the last years, several SynBio platforms have been established, such as Golden Gate (Engler and Marillonnet, 2014), GoldenBraid (GB) (Sarrion-Perdigones et al., 2011), MoClo (Weber et al., 2011), and FungalBraid (FB), with the latter being a GB variant tailored to filamentous fungi and developed by our group (Hernanz-Koers et al., 2018; Vazquez-Vilar et al., 2020; Moreno-Giménez et al., 2023). FB makes use of pCAMBIA-derived binary vectors for ATMT with the great advantage of their interchangeability between plants and fungi as long as they are functionally compatible (Hernanz-Koers et al., 2018; Moreno-Giménez et al., 2023).

To date, several strategies have been applied in *A. vadensis* to further improve this filamentous fungus as a host for homologous and heterologous protein production (Culleton et al., 2014a; Culleton et al., 2014b; Ourdia and de Vries, 2014). However, this fungus has only been genetically modified through PMT and classical cloning strategies so far, which decreases the exploitation capacity of this fungus at the industrial level. In this study, we have applied the FB system and developed an ATMT protocol for *A. vadensis* for the first time. As a case study, we have successfully complemented and fluorescently tagged a uridine auxotrophic *A. vadensis pyrA*$^-$ strain and generated *A. vadensis* strains carrying the *Penicillium expansum*-based expression cassette for the heterologous production of the antifungal protein PeAfpA from *P. expansum* (Garrigues et al., 2018; Gandía et al., 2022). Overall, this study will be of great interest for the scientific community to further aid the exploitation of *A. vadensis* as fungal workhorse for the production of homologous and heterologous proteins for multiple biotechnological applications.

2. Material and methods

2.1. Strains, media and growth conditions

The fungal strain *A. vadensis* CBS 113226 (*pyrA*$^-$) (de Vries et al., 2004), which derives from *A. vadensis* CBS 113365 (wild type), was used as parental strain for subsequent transformations. Fungi were routinely cultured at 30 °C in Potato Dextrose Agar (PDA, Difco-BD Diagnostics, Sparks, US) supplemented with 1.22 g/L uridine (Sigma-Aldrich, St. Louis, US) when required. After 5–7 days of growth, conidia were harvested, dispersed in sterile dH_2O and concentration was adjusted using a haemocytometer. For growth analyses of *A. vadensis* mutants, 500 conidia were deposited on the center of *Aspergillus* Minimal Medium (MM) plates (de Vries et al., 2004), MM supplemented with 1.22 g/L uridine, and MM supplemented with 1.22 g/L uridine and 1.25 g/L 5-Fluoroorotic acid (5′-FOA, Formedium Ltd., Hunstanton, England), and colony morphology was assessed and compared by visual inspection.

Plasmids were propagated in *Escherichia coli* JM109 grown in Luria Bertani (LB) medium supplemented with either 25 μg/mL chloramphenicol, 50 μg/mL kanamycin or 100 μg/mL spectinomycin at 37 °C depending on the vector (pUPD2, pDGB3α or pDGB3Ω, respectively).

A. tumefaciens AGL-1 strain was grown in LB agar medium supplemented with 20 μg/mL rifampicin at 28 °C for 48 h and was used for fungal transformation.

2.2. Construction of binary vectors

All binary vectors previously available or generated in this study were obtained according to the FB/GB rules and tools (https://gbcloning.upv.es) and have been listed in Table 1. Briefly, DNA parts (promoters, coding sequences (CDSs), terminators) were domesticated to eliminate *Bsa*I and *Bsmb*I cutting sites and were ligated into the pUPD2 entry vectors via the restriction-ligation protocol as described (Hernanz-Koers et al., 2018; Vazquez-Vilar et al., 2020). Multiple assemblies into pDGB3α vectors of the DNA parts contained in pUPD2 vectors were carried out to obtain the different transcriptional units (TUs), and binary assemblies were subsequently performed to combine the different TUs into multigenic constructs (e.g., together with selection markers) within these pDGB3α or pDGB3Ω vectors as previously described (Hernanz-Koers et al., 2018; Vazquez-Vilar et al., 2020; Moreno-Giménez et al., 2023). Positive clones were confirmed by restriction analyses and/or by PCR analyses using NZYTaq II DNA polymerase (nzytech, Lisbon, Portugal). All binary vectors generated that would later be used for fungal transformation (FB293, FB376 and FB401) were first introduced into *A. tumefaciens* AGL-1 via electroporation. After electroporation, antibiotic resistant colonies of AGL-1 were grown in LB supplemented with the corresponding antibiotic (kanamycin in case of pDGB3α or spectinomycin for pDGB3Ω) at 28 °C and 200 rpm for 48 h. Plasmids were isolated (NZYMiniprep kit, nzytech) and confirmed through restriction analyses. Clones containing the positive plasmids were (1) glycerinated and (2) streaked into LB agar plates. A single positive colony of AGL-1 was subsequently used for fungal transformation.

2.3. Optimization of the ATMT method for A. vadensis

The ATMT method for the auxotrophic *A. vadensis* CBS 113226 (*pyrA*$^-$) was adapted from previously described procedures (Vazquez-Vilar et al., 2020). Optimization of the ATMT method was accomplished using the *A. tumefaciens* AGL-1 strain that harbors the FB293 binary vector, which contains *pyr4* TU that would restore original *A. vadensis* uridine/uracil auxotrophy (Table 1). Conditions tested for optimization included (1) fungal spore concentration to be transformed (10^6, 10^7 and 10^8 conidia/mL), (2) fungus/bacterium co-cultivation temperature (20, 24 and 28 °C) and (3) co-cultivation time (48 and 60 h). Optimization

Table 1
FB plasmids used in this study.

Code	Genetic element/ Assembly	Plasmid	Description	Reference
FB026	P*gpdA*::*yfp*::T*trpC*	pDGB3α1R	Assembly of the transcriptional unit of *yfp*	(Hernanz-Koers et al., 2018)
FB112	P*afpA*::*afpA*::T*afpA*	pDGB3α1R	Assembly of the transcriptional unit of *afpA*	(Gandía et al., 2022)
FB293	P*pyr4*::*pyr4*::T*pyr4*	pDGB3α2	Assembly of the transcriptional unit for the auxotrophy marker *pyr4* from *T. reesei*	(Moreno-Giménez et al., 2023)
FB376	FB026 + FB293	pDGB3Ω1	Binary assembly to express *yfp* in *A. vadensis*	This study
FB401	FB112 + FB293	pDGB3Ω1	Binary assembly to express *afpA* in *A. vadensis*	This study

assays were performed with three replicates per condition tested.

Once optimized, the protocol was applied as shown in Fig. 1. For each transformation, *A. tumefaciens* AGL-1 strain containing the desired binary vector was inoculated into 10 mL of transformation Minimal Medium (tMM) supplemented with either 50 μg/mL kanamycin or 100 μg/mL spectinomycin (depending on the vector) and incubated for 48 h at 28 °C and 250 rpm. After incubation, 1 mL of AGL-1 culture was centrifuged for 10 min at 1,000 × g. The pellet was resuspended in 1 mL of Induction Medium (IM) supplemented with either 50 μg/mL kanamycin or 100 μg/mL spectinomycin and 400 μM acetosyringone. The suspension was added to 4 mL IM medium in a 100 mL Erlenmeyer flask to reach a final volume of 5 mL. The cultures were then incubated at 250 rpm and 28 °C to yield a final optical density (OD) at $\lambda = 600$ nm (OD_{600}) of 0.6 (~1×10^8 bacterial cells/mL). Then 100 μL of this culture were mixed with 100 μL of *A. vadensis* conidia at an initial concentration of 10^8 conidia/mL. Aliquots (200 μL) were spread over four to six sterile 0.45 μM nitrocellulose filters (Sartorius, Göttingen, Germany) placed onto 50 mm diameter Petri dishes of co-culture medium (CM) supplemented with kanamycin/spectinomycin, acetosyringone and 1.22 g/L uridine. Plates were incubated in the darkness for 3 days at 24 °C. Membranes were then transferred onto MM selection plates (50 mm diameter) supplemented with the corresponding antibiotic and 200 μM cefotaxim and 100 μg/mL moxalactam to kill *A. tumefaciens*. After 24 h incubation at 30 °C, selection plates were washed by adding 1.2 mL sterile H_2O onto the plates. Between 250 and 300 μL of the resultant spore suspensions (equivalent to a 1:4 dilution) were finally plated in MM selection plates (90 mm diameter). After 6–7 days of growth at 30 °C, visible transformants were identified and selected for further confirmation. Transformation with the FB293 binary vector was always included as a positive control in each assay and a mock transformation without adding AGL-1 strain during the co-cultivation step was included as a negative control.

All media compositions used for *A. vadensis* transformation are described in Supp. Table S1 and Supp. Table S2.

2.4. Selection and confirmation of transformant strains

Viable transformants were selected and transferred into MM medium in 24-well microtiter plates (Nunc, Rosklide, Denmark). After 2–3 days of growth at 30 °C, spores were recovered and streaked into PDA plates for monosporic culture. For molecular characterization, spores were simultaneously inoculated into Potato Dextrose Broth (PDB, Difco-BD Diagnostics) medium for mycelia growth during 2 days at 30 °C and 150 rpm. Genomic DNA was then isolated as previously described (Gandía et al., 2014). Positive transformants were confirmed by PCR amplification of genomic DNA using specific primers designed to discriminate each FB cassette (Table 2).

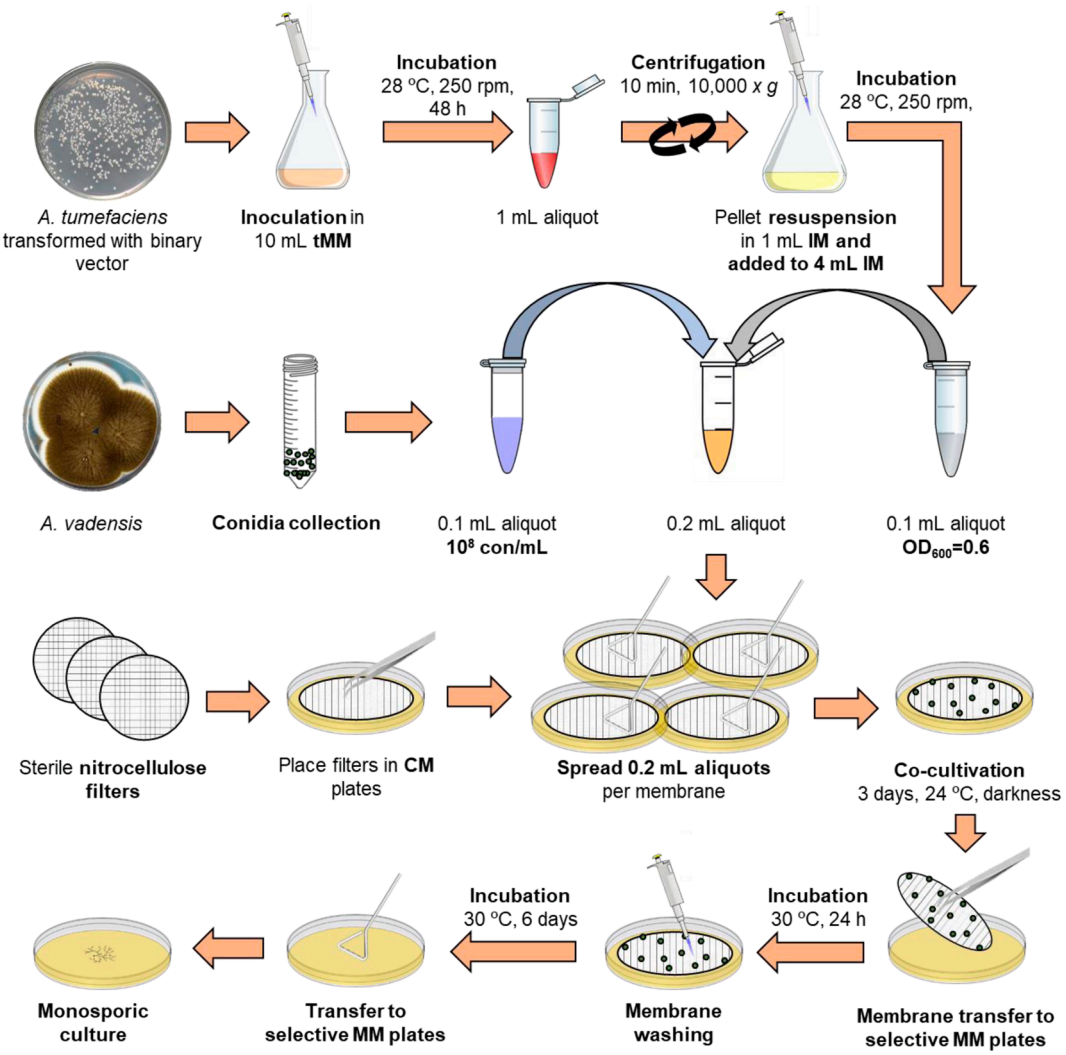

Fig. 1. Schematic overview of *A. tumefaciens*-mediated transformation (ATMT) of *A. vadensis*. See main text for details.

Table 2
Primers used in this study.

Primer	Use	Sequence 5′-3′	Tm	Origin	Reference
OJM509	F	GCGCCGTCTCGCTCGGGAGTGGCGCATGCGGACAGACGG	64	P*gpdA*	(Hernanz-Koers et al., 2018)
OJM522	R	GCGCCGTCTCGCTCAAGCGCATGTCTCAGACGGTCGATG	62	T*trpC*	(Hernanz-Koers et al., 2018)
OJM620	F	GCTTGTCCGGCCTTGTGACGCC	67	P*afpA*	(Gandía et al., 2022)
OJM621	R	AGGAAGGCCCCGAGGAAGATGC	67	T*afpA*	(Gandía et al., 2022)
OJM696	F	TTGTCTCACTCTCTCTTTTCC	55	*pyr4*	(Moreno-Giménez et al., 2023)
OJM697	R	ATTCCATGCTTCCAGATCC	55	*pyr4*	(Moreno-Giménez et al., 2023)

2.5. Fluorescent microscopy assays

For the visualization of fluorescence in *A. vadensis* hyphal cells, suspensions of 5×10^4 spores/mL were grown in 100 μL PDB (supplemented with 1.22 g/L uridine for the parental CBS 113226) onto autoclaved coverslips for 24 h at 28 °C. After this, the mycelium was washed, fixated and suspended in 50 % glycerol as described (Garrigues et al., 2016). For spore visualization, conidia solutions of 1×10^8 spores/mL were used. Confocal images were obtained with a FV1000 confocal laser microscope (Olympus, Tokyo, Japan). YFP fluorescence (emission peak at $\lambda = 527$ nm) was observed by illuminating with a 515 nm laser. Bright-field images were captured with a transmitted light detector. Objective used was a UPLFLN40X oil, combined with a zoom of 1x/3x. Main Z-stack confocal image properties were 2 channels, 16 bits, frame size of 1024 x 1024 pixels. Specific properties for "3z" stacks were 105 x 105 μm as the size of field of view (pixel size is 0.103 μm/px) and a 0.5 μm Z-step value. Specific properties for "1z" stacks were 317 x 317 μm as the size of field of view (pixel size is 0.103 μm/px) and a 1 μm Z-step value. Average brightness projections for fluorescent images were executed with Fiji software (Schindelin et al., 2012).

2.6. Protein production assays

For protein production analysis, 10^6 conidia/mL were inoculated into 25 mL of *Aspergillus* MM or Complete Medium (CM) (Supp. Table S2) (de Vries et al., 2004) supplemented with 2 % (wt/vol) D-glucose or sucrose as the sole carbon source. Incubation was carried out at 30 °C and 150 rpm, and growth was inspected daily until 11 (for MM) or 7 days (for CM). Supernatants were collected at different culture times to determine protein production through SDS-PAGE and western blot analysis.

Total proteins from supernatants and pure PeAfpA protein (purified as previously described (Garrigues et al., 2018)) were separated by SDS-PAGE (16 % polyacrylamide gels) and transferred to Amersham Protran 0.20 μm NC nitrocellulose transfer membrane (GE Healthcare, Freiburg, Germany) as previously described (Garrigues et al., 2018). Immunodetection was accomplished using 1:2500 anti-PeAfpA antibody, and 1:20000 of ECL NA934 horseradish peroxidase donkey anti-rabbit (GE Healthcare) was used as the secondary antibody. Chemiluminescent detection was then performed with ECLTM Select Western blotting detection reagent (GE Healthcare) and images were captured with Amersham Imager 680 (GE Healthcare).

2.7. Antimicrobial activity assays

Antifungal activity assays were conducted in 96-well flat-bottom microtiter plates (Nunc) as described in (Garrigues et al., 2017) with minor modifications. Briefly, 50 μL of 10 % PDB or 100 % MM both supplemented with 2.5 mg/mL uridine and 0.02 % (wt/vol) chloramphenicol containing 2 × conidia solution (5×10^4 conidia/mL) were mixed in each well with 50 μL of 2 × protein solutions from serial 2-fold dilutions (final PeAfpA concentrations ranging from 0.125 to 8 μg/mL in PDB and 1 to 64 μg/mL in MM). Plates were incubated at 30 °C and growth was determined daily by measuring the OD_{600} using a FLUOstar Omega plate spectrophotometer (BMG labtech, Orlenberg, Germany). Dose-response curves were generated from measurements after 72 h and the Minimum Inhibitory Concentration (MIC) was defined as the protein concentration that completely inhibited growth in all the experiments performed.

2.8. Statistical analysis

For the calculation of transformation efficiency by ATMT, experiments were conducted in triplicate and significant differences were determined with one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test ($p < 0.05$) using SPSS software (version 28.0.1.0).

3. Results and discussion

3.1. The ATMT system has been established in A. vadensis

In this study, we have implemented a transformation protocol to genetically modify *A. vadensis* CBS 113226 (*pyrA*$^-$) through ATMT for the first time (Fig. 1). In addition, we have genetically modified *A. vadensis* by means of SynBio through the FB system. Previously, only PMT and classical cloning methods allowed the transformation and genetic modification of this species (Culleton et al., 2014a). However, compared to the protoplast method, ATMT is simpler because it directly uses fungal spores and omits several tricky steps related to protoplast manipulation. Furthermore, it is relatively easy to generate stable transformants and high homologous recombination frequencies are obtained via ATMT (Michielse et al., 2005). Therefore, fungi with a long record of PMT, such as *Aspergillus nidulans, A. niger* or *A. oryzae* are starting to be transformed via ATMT (Nguyen et al., 2016; Casado-del Castillo et al., 2021; Thai et al., 2023). In addition, the implementation of the SynBio FB platform to *A. vadensis* opens the door to accelerate the study and exploitation of this species as cell factory at the biotechnological and industrial level.

Critical factors affecting ATMT efficiency include the spore concentration, the composition of the induction medium, and time intervals and temperatures for co-cultivation (de Groot et al., 1998; Michielse et al., 2005; Nguyen et al., 2016). Here, optimization of the ATMT method for *A. vadensis* was achieved after contrasting: (1) fungal spore concentration to be transformed (10^6, 10^7 and 10^8 conidia/mL); (2) fungus/bacterium co-cultivation temperature (20, 24 and 28 °C) and (3) co-cultivation time (48 and 60 h) (Fig. 2). We used FB293, a FB binary vector previously generated in our laboratory that contains the orotidine 5′-phosphate decarboxylase-encoding *pyr4* TU (Moreno-Giménez et al., 2023), as a test case aimed to restore the parental uridine auxotrophy (Fig. 3A, Table 1). This vector contains the *Trichoderma reesei pyr4* gene, an ortholog of the *Aspergillus pyrA/pyrG* that has been widely applied as a strong auxotrophic selection marker (Derntl et al., 2015), and would allow us to easily identify *A. vadensis* prototrophic transformants on uridine-free plates. The FB binary vector FB293 is also available through Addgene (https://www.addgene.org/203613/).

Our results revealed that the fungus/bacterium co-cultivation temperature was the main factor determining the success of the ATMT method in *A. vadensis*. Initially, 28 °C was set as this is the optimal for both *A. vadensis* and *A. tumefaciens* growth. However, this temperature gave no or very few prototrophic transformants on the transformation

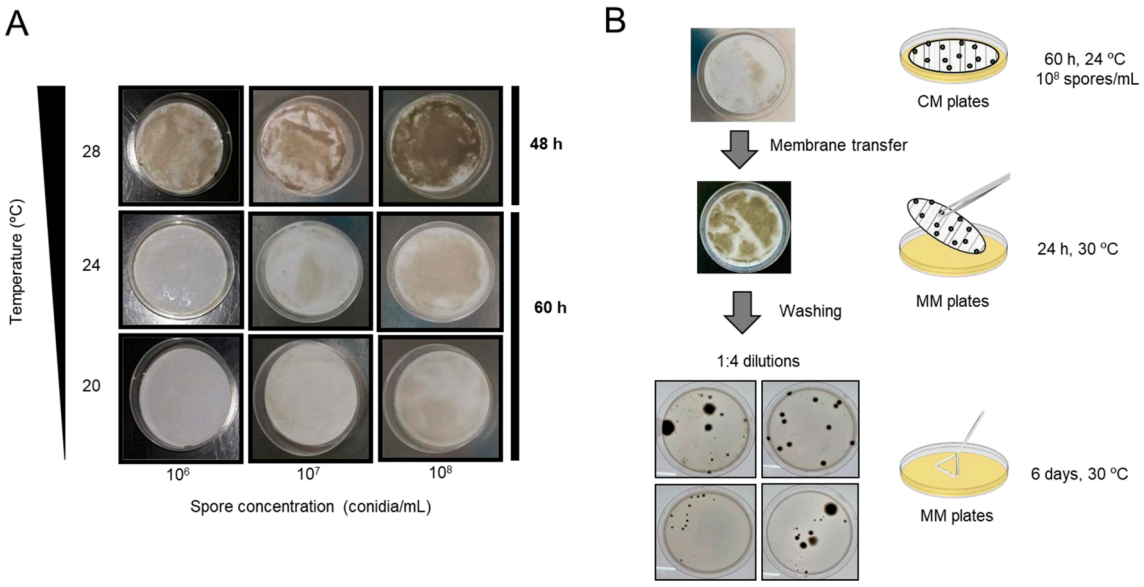

Fig. 2. ATMT protocol optimization. (A) Representative images of ATMT co-cultivation plates of *A. vadensis* CBS 113226 after incubation at different temperatures (20, 24 and 28 °C), fungal spore concentrations (10^8, 10^7 and 10^6 conidia/mL) and co-cultivation times (48 and 60 h). (B) Schematic representation of the ATMT protocol with the optimized conditions for *A. vadensis* transformation. Representative images of each step of the protocol are included as examples of the results obtained.

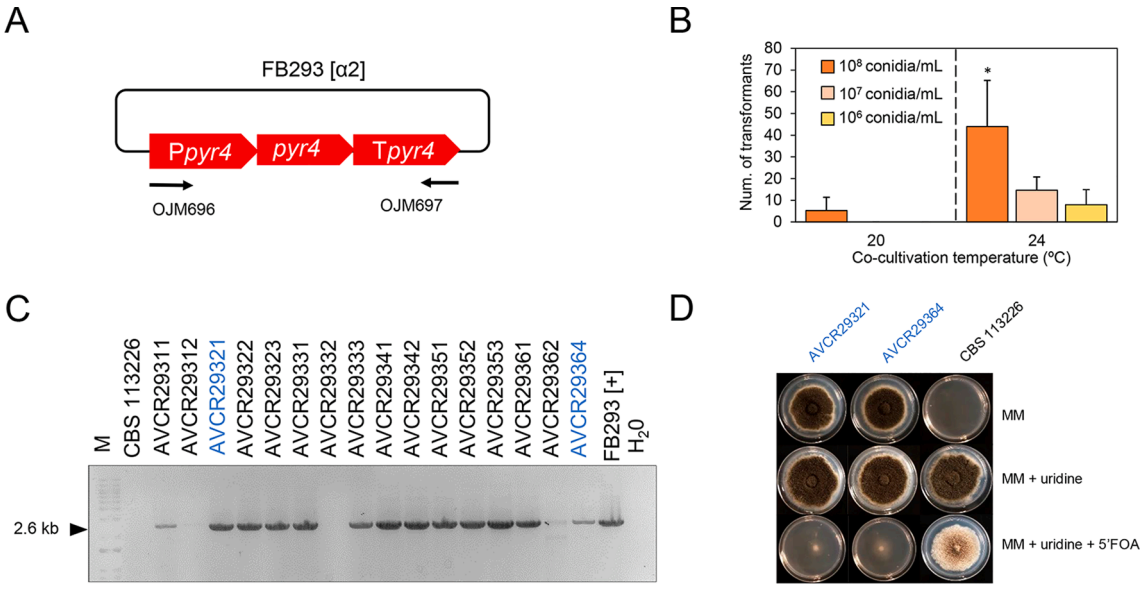

Fig. 3. *A. vadensis* transformation through ATMT with binary vector FB293. (A) Plasmid pDGB3α1 FB293 for the ectopic integration of *pyr4* TU in the auxotrophic *A. vadensis pyrA*- mutant (CBS 113226). (B) Effect of temperature and fungal spore concentration on *A. vadensis* transformation efficiency by ATMT. Co-cultivation time was set for 60 h in all cases. Experiments were conducted in triplicate and data are represented as mean ± standard deviation. Significant differences were determined with one-way ANOVA and Fisher's LSD test (*, $p < 0.05$). (C) Molecular characterization of the transformed strains with oligos OJM696 and OJM697 located as indicated in (A). The 2.6 kb band corresponds to the complete *pyr4* cassette. Selected strains are highlighted in blue. (D) Phenotypic characterization of *A. vadensis* prototrophic strains and the parental CBS 113226 after 7 days of growth at 30 °C in Minimal Medium (MM), MM supplemented with uridine and MM supplemented with uridine and 5-Fluoroorotic acid (5′FOA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

plates, with an average number of 3 ± 5 transformants per 10^5 spores after 48 h of co-cultivation period with 10^6 conidia/mL. Noteworthy, no transformed strains were obtained using higher conidia concentrations (>10^7 conidia/mL), therefore, demonstrating that there is no linear correlation in the fungal/bacterial ratios used in ATMT transformation. It has been described that the *A. tumefaciens* T-DNA transfer machinery is greatly sensitive to temperatures above 26 °C, which might partially explain these low efficiencies of transformation (Fullner and Nester, 1996; Baron et al., 2001). Remarkably, temperature of 28 °C promoted *A. vadensis* over-growth during co-cultivation at all fungal spore concentrations tested (Fig. 2A), suggesting that an improper bacterium/conidia ratio could be hampering the T-DNA transfer and the subsequent generation of transformants.

To avoid fungal over-growth on the co-cultivation plates, lower temperatures (20 and 24 °C) of co-cultivation were then evaluated. In these cases, co-cultivation time was parallelly expanded from 48 to 60 h

to promote fungal growth while facilitating bacteria/conidia interaction. Reducing the temperature to 20 °C considerably suppressed background growth (Fig. 2) but failed to yield more transformants in the selection plates. At 20 °C, an average number of 5 ± 6 transformants per 10^7 spores was obtained after co-cultivation with a conidia concentration of 10^8 spores/mL and no prototrophic strains were identified with lower conidial titers (Fig. 3B). On the contrary, setting 24 °C as the temperature of choice for fungal/bacterial co-cultivation resulted in a higher number of *A. vadensis* transformants on the transformation plates, with the maximum number of transformants reached at a fungal concentration of 10^8 conidia/mL (Fig. 3B). Therefore, transformation with 10^8 conidia/mL and co-cultivation at 24 °C for 60 h were selected as the standard parameters for ATMT in *A. vadensis* (Fig. 2B), which, even though are not the parameters that would give the highest efficiency of transformation in terms of transformants per total spores, are the best conditions that gave the highest amount of prototrophic strains on the plates, which allowed us to continue with transformant isolation and characterization.

Results after four independent transformations assays with the chosen parameters showed an average efficiency of 32 ± 17 transformants per 10^7 spores. Several colonies were randomly picked in each of the four transformation experiments to molecularly confirm the presence of the *pyr4* cassette in the genome. As a result, a total of 90 ± 13 % of the selected strains contained the *pyr4* cassette (see Fig. 3C as an example of an independent transformation event).

Although the transformation efficiency obtained for *A. vadensis* through ATMT is lower than that reported for other filamentous fungi such as *Fusarium oxysporum* (300–500 transformants per 10^6 spores) (Mullins et al., 2001), *Aspergillus flavus* (60 transformants per 10^6 spores) (Han et al., 2018), *Trichoderma reesei* (10–20 transformants per 10^5 conidia) (Zhong et al., 2011) or *Aspergillus awamori* (183 ± 14 transformants per 10^6 spores) (Michielse et al., 2004), our results are in concordance with that obtained via ATMT for its close relative *A. niger* (83 transformants per 10^7 spores) (Li et al., 2013) and for other filamentous fungi such as *A. oryzae* (18–20 transformants per 10^7 spores) (Nguyen et al., 2016). Thus, even though there is still room for improvement, the ATMT transformation protocol described here for the first time for *A. vadensis* has shown to successfully generate a proper handling number of transformants with a genomic modification efficiency between 80 and 100 %.

3.2. *pyr4* successfully restored the uridine auxotrophy of *A. vadensis pyrA⁻* strain

So far, *T. reesei pyr4* gene has been widely applied as a strong auxotrophic selection marker that can be counter-selected using 5′FOA in fungi from distinct phylogenetic classes, from Sordariomycetes (*Trichoderma* sp., *Neurospora* sp.) to Ascomycetes (*Aspergillus* sp., *Penicillium* sp.) (Ballance and Turner, 1985; Díez et al., 1987; Gruber et al., 1990). After ATMT of *A. vadensis*, several transformants which could grow in the absence of uridine were chosen for PCR analysis to confirm the *pyrA::pyr4* complementation. From the sixteen transformants analysed in an independent transformation assay (Fig. 3C), fourteen showed the presence of the *pyr4* TU in the genome (87.5 % efficiency). From the confirmed positive transformants, two (AVCR29321 and AVCR29364, Fig. 3C in blue) were randomly chosen to undergo phenotypic characterization and further verify the *pyr4* functionality in this fungal chassis. As shown in Fig. 3D, complemented mutants were able to grow on uridine-free MM plates, in contrast to the parental CBS 113226. Furthermore, *pyr4* complemented mutants failed to grow in the presence of 5′-FOA, as expected for prototrophic strains. These results demonstrate not only the suitability of the ATMT method to obtain genetically modified *A. vadensis* strains, but also confirm the functionality of FB293 as an auxotrophic selection marker in fungal species other than *Penicillium*, since this TU had been only functionally validated in the phytopathogenic fungus *Penicillium digitatum* to date (Moreno-Giménez et al., 2023).

3.3. Fluorescent tagging of *A. vadensis*

Fluorescence trigger genes, such as the green, yellow and red fluorescent proteins (GFP, YFP, RFP, respectively) are often used as reporters for the visualization of gene expression and protein subcellular localization in different microbial species, allowing fluorescence microscopy of live cells with minimal perturbation (Sheff and Thorn, 2004). In this work, we carried out ATMT of *A. vadensis* for the generation of YFP-tagged strains. For this aim, we used the FB binary vector FB376 (Table 1) that resulted from the assembly of the previously described functional TU for *yfp* expression (FB026) and the TU for *pyr4* expression (FB293) (Fig. 4A). The functionality of FB026 was previously validated in fungi from different genera, driving intense green fluorescence in *P. digitatum*, *P. expansum,* and *A. niger* (Hernanz-Koers et al., 2018; Vazquez-Vilar et al., 2020). Therefore, we also expected *yfp* to drive green fluorescence in *A. vadensis*.

After ATMT transformation, thirteen transformants -which could grow in the absence of uridine in the culture medium due to the presence of *pyr4*- were chosen for PCR analysis to confirm the presence of the *yfp* expression cassette (Fig. 4B). From the thirteen transformants analysed, all showed the presence of the *yfp* TU in the genome (100 % efficiency). From the confirmed positive transformants, two were randomly chosen for phenotypic characterization using fluorescence microscopy (AVCR37631 and AVCR37642, Fig. 4B in blue). Both mutants displayed intense green cytosolic fluorescence homogeneously distributed in both spores (Fig. 4C) and hyphae (Fig. 4D), thus confirming the suitability of the recently implemented ATMT method and the SynBio FB system for the generation of fluorescently labelled *A. vadensis* strains that could be interesting for subsequent functional analyses of biotechnological relevance.

3.4. Suitability of *A. vadensis* as biofactory for the heterologous production of antifungal proteins

A. vadensis has been suggested as a more favourable alternative to the widely exploded industrial workhorse *A. niger* for both homologous and heterologous protein production due to its low protease activity and its inability to acidify the culture media (de Vries et al., 2004; Culleton et al., 2014a). In addition to this, *A. vadensis* does not produce the enzymes required to degrade the storage polysaccharide starch (Culleton, 2015), being this strain a naturally occurring low-background host.

In this study, we applied ATMT and the FB system to the fungus *A. vadensis* to assess its suitability for the heterologous production of the so-called fungal antifungal proteins (AFPs). AFPs are small, cationic, cysteine-rich proteins that are usually secreted by filamentous ascomycetes and have potent antifungal activity against opportunistic human, animal, plant, and foodborne pathogenic fungi at the micromolar range (Hegedüs and Marx, 2013; Delgado et al., 2016; Garrigues et al., 2017; Garrigues et al., 2018; Martínez-Culebras et al., 2021), which makes them promising alternatives for the development of novel antifungals. One of the most active AFPs in literature is the antifungal protein PeAfpA from the phytopathogenic fungus *P. expansum* (Garrigues et al., 2018; Gandía et al., 2020). The *P. expansum* CECT 20906 strain naturally produces up to 125 mg/L of PeAfpA in the culture supernatants. However, the main concern about *P. expansum* is its mycotoxin-producing nature (Tannous et al., 2018), which hampers its application. In this sense, the non-mycotoxigenic, low-protease background *A. vadensis* would be a better biofactory alternative to *P. expansum* with high potential for the safe production of PeAfpA. To assess this, we generated the pDGB3Ω1 vector FB401 carrying the *pyr4* TU assembled with the *P. expansum*-based expression cassette -which contains the *afpA* promoter, signal peptide for secretion, gene and terminator sequences- (Gandía et al., 2022) (Fig. 5A, Table 1) and ectopically transformed it by ATMT in the auxotrophic *A. vadensis* CBS 113226 (*pyrA⁻*). After the

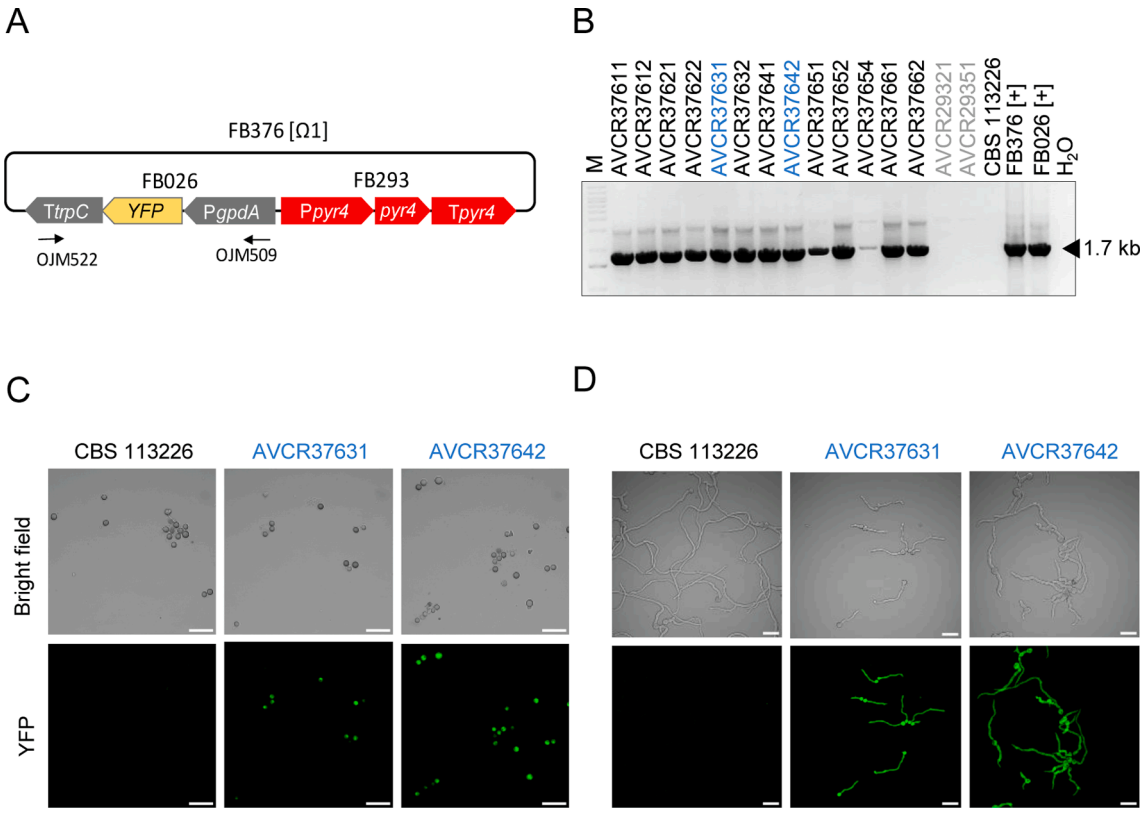

Fig. 4. Generation of fluorescently labelled *A. vadensis* strains through ATMT and FB. (A) Schematic diagram of the binary assembly of vector FB372 containing the auxotrophic selection marker *pyr4* (FB293) and the *yfp* expression cassette (FB026). Oligos OJM522 and OJM509 were used for the molecular characterization of the transformed strains shown in (B). The 1.7 kb band corresponds to the *yfp* cassette. Representative strains chosen are highlighted in blue. Bright field and fluorescent images of fungal spores (C) and mycelium (D) of the parental *A. vadensis* CBS 113226 and the two representative transformants (AVCR37631 and AVCR37642) chosen from (B). Bars correspond to 15 μm in (C) and 30 μm in (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genetic transformation, fifteen *A. vadensis* transformants were characterized by PCR. As shown in Fig. 5B, all the selected transformants successfully carried the *P. expansum*-based expression cassette (100 % efficiency). From these fifteen, five independent mutants (AVCR40111, AVCR40121, AVCR40131, AVCR40141 and AVCR40151) were chosen and tested for PeAfpA production in different culture media (Fig. 5C). Transformant AVCR29321 was included as a reference of a non-producing prototrophic strain. As shown in the SDS-PAGE and western blot analyses, no PeAfpA production was detected in either MM or CM (with either glucose or sucrose as sole carbon source) after 11 or 7 days of growth, respectively (Fig. 5C). These surprising results could be explained either by (i) the non-induction of the *afpA* expression cassette in the two media analysed; (ii) the non-functionality of *afpA* regulatory sequences in *Aspergillus* species; or (iii) the putative high antifungal activity being exerted by PeAfpA against *A. vadensis*.

Even though this is not the first time an AFP protein is not produced in fungi regardless of gene expression, as it is the case of *Penicillium digitatum* AfpB (Garrigues et al., 2016) or *P. chrysogenum* PAFB (Huber et al., 2018), minimal medium has been the medium of choice for the production of PeAfpA in *P.* expansum (Garrigues et al., 2018) and has been reported to induce the *P. expansum*-based expression cassette in *Penicillium* species (Gandía et al., 2020). However, it could be the case that for *A. vadensis* different medium composition with more complex carbon sources is required to trigger the induction of *afpA* promoter, as reported for the production, for instance, of AFP from *Aspergillus giganteus* (Lacadena et al., 1995), PgAFP and PAFB from *P. chrysogenum* (Delgado et al., 2015; Huber et al., 2019), or AnAFP from *A. niger* (Gun Lee et al., 1999) in their native fungi. Therefore, different culture media will be evaluated in the near future to try to trigger PeAfpA production in *A. vadensis*.

Another aspect to be taken into account is that the *P. expansum*-based expression cassette used in this study has not been validated in *Aspergillus* species so far, opening the door to the need to optimize this expression cassette for the genus *Aspergillus*. In the case of *A. vadensis*, several promoter sequences have been validated to improve heterologous protein production in this strain, being the constitutive promoters from the elongation factors 1α (P*ef1α*) and 1β (P*ef1β*) from *A. niger*; and the glyceraldehyde-3-phosphate dehydrogenase promoter (P*gpda*) and a mitochondrial ATP synthase promoter (P*oliC*) from *A. vadensis* the ones resulting in higher heterologous protein production in this species (Culleton et al., 2014b). The development of an *Aspergillus*-based expression system by using the *A. niger* anAFP regulatory sequence (Wanka et al., 2016) to drive *afpA* expression in *A. vadensis* would also be an alternative approach to see if an *Aspergillus* AFP promoter works better than a *Penicillium* AFP promoter for triggering PeAfpA production in this biofactory.

Finally, another possibility that would explain the non-production of PeAfpA would be due to the antifungal activity exerted by this protein against *A. vadensis*, which would prevent the fungus from producing and secreting it into the culture medium. To evaluate this hypothesis, antimicrobial activity assays were performed and dose–response curves of PeAfpA against *A. vadensis* were obtained. As shown in Fig. 5D, PeAfpA has a potent inhibitory activity against *A. vadensis* in both nutrient-rich and minimal media with a MIC value of 1 μg/mL in PDB and 2 μg/mL in MM. However, these results do not necessarily discard the possibility to use *A. vadensis* as biofactory for AFP production, since other PeAfpA-sensitive fungi such as *P. chrysogenum* (MIC_{PeAfpA} = 1 μg/mL) produce PeAfpA in the culture supernatants (Garrigues et al., 2018; Gandía et al.,

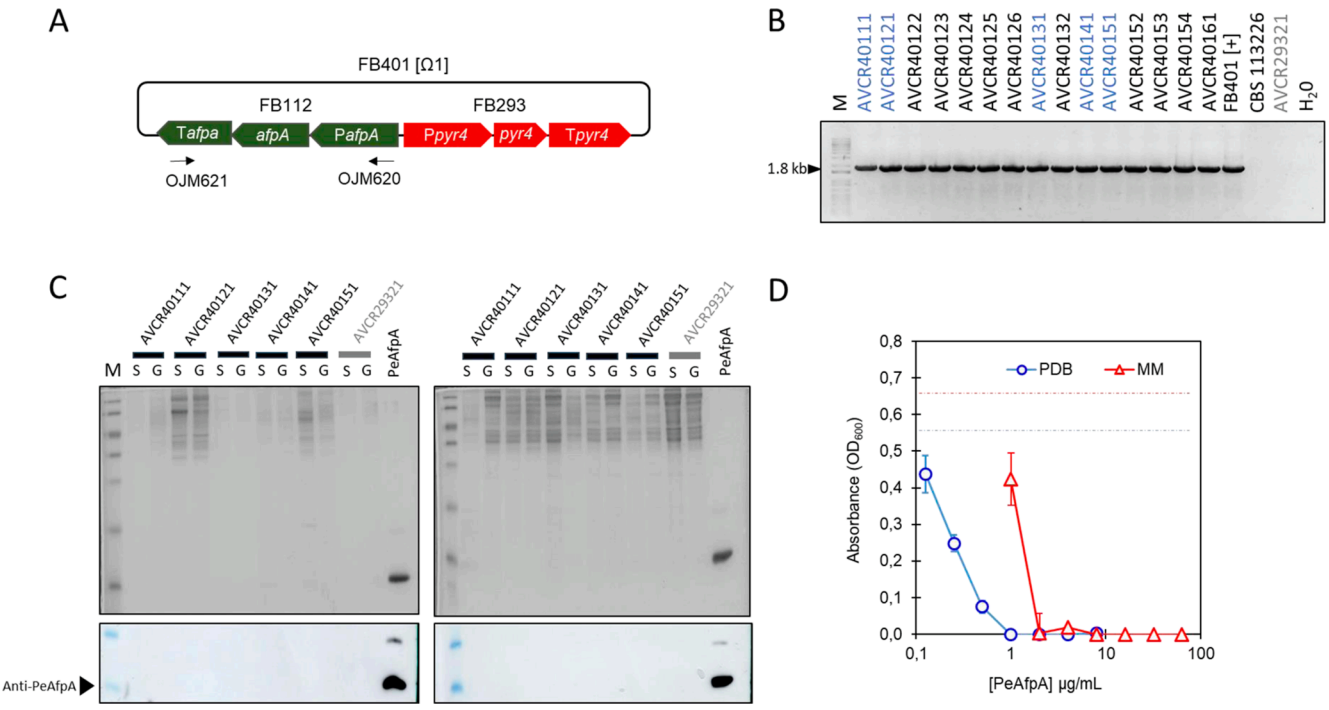

Fig. 5. Confirmation of *A. vadensis* mutants carrying FB401. (A) Schematic representation of plasmid pDGB3Ω1 FB401 for the ectopic integration of the *P. expansum*-based expression cassette for PeAfpA production in the auxotrophic *A. vadensis* CBS 113226. Oligos OJM620 and OJM621 were used for molecular characterization of the transformants described in (B). The 1.8 kb band corresponds to the complete *afpA* gene. Selected strains are highlighted in blue. (C) SDS-PAGE (top) and western blot analyses (bottom) of *A. vadensis* transformants for PeAfpA production (10 µL of 10 × culture supernatants loaded per lane) after growth in Minimal Medium (MM, left pannel) and in Complete Medium (CM, right pannel) supplemented with 2 % glucose (G) or sucrose (S). Transformant AVCR29321 was included as a reference of a non-producing prototrophic strain and 1 µg of pure PeAfpA was loaded as control. M: SeeBlue® pre-stained protein standard. (D) Dose–response curves of PeAfpA against the parental *A. vadensis* CBS 113226. Data are mean values ± SD of triplicate samples after 72 h of growth in MM and PDB at 30 °C. Dashed lines represent control growth value in the absence of the protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2022).

Although we have not found the optimized conditions for AFP production in *A. vadensis* yet, this species has been already applied to successfully produce industrially relevant enzymes and other proteins, such as the feruloyl esterases FaeB from *A. niger* and FaeA from *Aspergillus tubingensis*, with yields higher than those of the parental strains (de Vries et al., 2004; Alberto et al., 2009). Additionally, *A. vadensis* produced one secreted arabinofuranosidase from *F. oxysporum* (Culleton et al., 2014b) and even one glucuronoyl esterases from the white-rot basidiomycete fungus *Phanerochaete chrysosporium* that could not be produced in *A. niger* (Duranová et al., 2009), highlighting the suitability of this strain as cell factory for reluctant proteins or proteins from phylogenetically distant species. We will try to find the condition(s) that best trigger PeAfpA production in *A. vadensis* in the near future. In this context, the implementation of the ATMT method in this species reported in this study, along with the utilization of the SynBio FB platform will greatly facilitate this task in a cost- and time-efficient manner.

4. Conclusions

In conclusion, we have demonstrated the successful implementation of an ATMT protocol for the genetic modification of the filamentous fungus *A. vadensis* by the generation of (i) uridine-prototrophic strains; (ii) fluorescently tagged strains; and (iii) mutant strains carrying the *P. expansum*-based expression cassette by using SynBio tools such as the FB platform. With this work, we expand the repertoire of methods and tools that allow the genetic manipulation of this biotechnologically relevant fungal strain and, therefore, contribute to accelerating the study and exploitation of the potential of *A. vadensis* as cell factory at the industrial level. Remarkably, results out of this work additionally validate the orthogonality of the DNA pieces in the SynBio FB system and deposited in Addgene, increasing the possibilities for the exploitation of filamentous fungi as cell factories.

CRediT authorship contribution statement

Carolina Ropero-Pérez: Investigation, Formal analysis, Writing – original draft. **Paloma Manzanares:** Supervision, Funding acquisition, Writing – review & editing. **Jose F. Marcos:** Supervision, Funding acquisition, Writing – review & editing. **Sandra Garrigues:** Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data is available through the DIGITAL CSIC repository (https://doi.org/10.20350/digitalCSIC/15707).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crbiot.2024.100178.

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