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Synthetic Biology Tools for Transcriptional Activation and Regulation of Biosynthetic Gene Clusters in Filamentous Fungi

László Mózsik

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Synthetic Biology Tools for Transcriptional Activation and Regulation of Biosynthetic Gene Clusters in Filamentous Fungi

PhD thesis

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Prelude

The number of discovered novel antimicrobials has drastically decreased after the “golden age of antibiotic discovery” (1950-1970). This decline can be explained by the high rate of known compound re-discovery as the main groups of antimicrobial natural products had already been discovered from culture-based assays and the high costs of clinical trials, which led to a diminished interest of pharmaceutical companies in funding novel antibiotic discovery research. The development of novel antimicrobials became so a neglected need, while the overuse of antibiotics resulted in a dramatic increase in microbial antibiotic resistance. To tackle this problem, among others, the Horizon 2020 European Union Programme for Research and Innovation funded numerous research programs in the field of antimicrobial resistance and identification of novel antimicrobials. One of such a project was the ALERT-COFUND Marie Skłodowska-Curie actions program between 2017 and 2021, where 19 research projects at the University of Groningen were co-funded in the field of antimicrobials, developing novel antimicrobial drug candidates by chemical, biotechnological and nanotechnological synthesis and the subsequent testing of promising candidates *in vivo* on relevant microbes. The objective of this thesis in the program was to develop novel synthetic biology tools for filamentous fungi, with the aim of transcriptional activation of transcriptionally silent and “cryptic” BGCs, as well as the controlled production of BGC encoded secondary metabolites in the fungal host.

Scope of the thesis

A surge of genomic data revealed that fungi are an excellent source for novel natural products in the form of transcriptionally silent biosynthetic gene clusters. Synthetic biology has revolutionized metabolic engineering with redesigning native genetic systems for various useful purposes. Various novel tools have been developed for different eukaryotic systems that can be applied for transcriptional regulation, but applications for filamentous fungi are lagging behind, despite their industrial relevance. This thesis therefore explores new tools for transcriptional activation of silent secondary metabolite gene clusters in filamentous fungi.

Chapter 1 represents an overview of regulation of secondary metabolite gene clusters in filamentous fungi, and lists currently available strategies to activate transcriptionally silent biosynthetic gene clusters.

Chapter 2 describes the construction of orthogonal, synthetic control devices for transcriptional regulation in *P. rubens*, using a hybrid transcription factor and various core promoters. The regulatory systems were evaluated using fluorescent reporters and the established system was applied for the control of penicillin production. The strength of the transcriptional control showed scalability by changing different modular elements of the expression system; as the strength of expression of the synthetic transcription factor, the number of upstream activating sequence (UAS) elements upstream in the synthetic promoter, and the chosen core promoter.

Chapter 3 described the construction of an *in vivo* expressed, episomal vector-based, CRISPR Cas9 delivery system for filamentous fungi. Several established fungal promoters were evaluated for the *in vivo* expression of CRISPR components in *P. rubens*. The system was validated using fluorescent reporter knockout experiments, and the established system was utilized for marker-free genome editing. The system was used to eliminate a recently identified

pseudo condensation domain in the L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine synthetase (ACVS) that mediates the formation of the tripeptide precursor for β-lactam antibiotics production.

Chapter 4 describes the development and application of a CRISPR-based transcriptional activation tool (CRISPRa) for regulation of transcriptionally silent genes in *P. rubens*. The CRISPRa elements (dCas9-VPR and the self-cleaved sgRNA) were delivered to *P. rubens* on a fungal episomal replicating vector, which is compatible with several filamentous fungal species, and its modular sgRNA insertion-site allows rapid library construction. The CRISPRa-based synthetic transcriptional activation tool was evaluated using fluorescent reporters and was applied for the activation of the transcriptionally silent macrophorin biosynthetic gene cluster. The CRISPRa system provided a rapid and convenient way for activation of transcriptionally silent genes, which can aid in the activation of cryptic biosynthetic gene clusters.

Chapter 5 presents an extensive tool for synthetic biology applications for filamentous fungi. The toolkit contains a collection of genetic elements which can be assembles in various combination, using the Golden Gate-based Modular Cloning assembly. The collection contains natural and synthetic promoters (constitutive and inducible), terminators, fluorescent reporters, and selection markers. Furthermore, there are regulatory and DNA-binding domains of transcriptional regulators and components for the construction of synthetic transcription factors or implementing different CRISPRa/i-based technologies. Genetic parts can be assembled into complex multipartite assemblies and delivered through genomic integration or expressed from an episomal AMA1-sequence-based, fungal-replicating shuttle vector. With this toolkit, synthetic transcription units with established promoters, fusion proteins, or synthetic transcriptional regulation devices can be more rapidly assembled in a standardized and modular manner for novel fungal cell factories.

CHAPTER 1

Transcriptional activation of biosynthetic gene clusters in filamentous fungi

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Abstract

Filamentous fungi are highly productive cell factories, many of which are industrial producers of enzymes, organic acids, and secondary metabolites. The increasing number of sequenced fungal genomes revealed a vast and unexplored biosynthetic potential in the form of transcriptionally silent secondary metabolite biosynthetic gene clusters (BGCs). Various strategies have been carried out to explore and mine this untapped source of bioactive molecules, and with the advent of synthetic biology, novel applications and tools have been developed for filamentous fungi. Here we summarize approaches aiming for the expression of endogenous or exogenous natural product BGCs, including synthetic transcription factors, assembly of artificial transcription units, gene cluster refactoring, fungal shuttle vectors, and platform strains.

Keywords: secondary metabolites, biosynthetic gene cluster, synthetic biology, synthetic transcriptional regulators, fungal platform strains

Introduction

Secondary metabolites (SM), commonly referred to as natural products, are chemical substances that are produced by living organisms, often bearing distinctive pharmacological effects. The exploitation of microorganisms for generating these valuable products for our societies in an economical manner has a great history. Notably, the use of filamentous fungi in industrial biotechnology is well established. With the introduction of synthetic biology, new tools and alternative methods are provided to further aid the metabolic engineering and exploitation of fungal workhorses. Filamentous fungi (with key players from *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, and *Neurospora* species) are highly efficient cell factories, often used industrially for the production of a diverse range of products such as proteins, enzymes, organic acids, and SMs.[1] SMs are not essential for the survival of the organism, but the production of these natural products often provides an evolutionary advantage. With the discovery of penicillin in 1928 produced by a mold identified as a *Penicillium* species, a new era started for industrial antibiotics production and the exploration and characterization of novel fungal SMs. This interest resulted in the discovery of not just antibacterial, but also antifungal (griseofulvin), cholesterol-lowering (lovastatin), immunosuppressant (cyclosporine), anticancer (paclitaxel), and food additive (carmine) compounds.[2] Alongside the beneficial metabolites, fungi also produce SMs acting as toxins (e.g. aflatoxin, fumonisin, patulin), which negatively affect food, feed, livestock, and human health.

Filamentous fungi are known prolific producers of SMs. The fungal kingdom currently consists of around 120,000 identified species, but this number is estimated to represent only 3-8% of the predicted number of existing species in our biosphere.[3] Thanks to next-generation and third-generation sequencing technologies, in recent years the number of publicly available genomes has grown tremendously. As of this moment (early 2022), there are several thousand fungal genomes deposited in public databases, e.g. more than 2000 only on Mycocosm, a project maintained by the Joint Genome Initiative (JGI).[4]

The simultaneous development of automated genome mining tools such as antiSMASH[5] and other bioinformatics tools[6,7] allowed researchers to identify a vast and unknown biosynthetic potential within the fungal kingdom in the form of SM encoding biosynthetic gene clusters (BGCs) (Figure 1a)[8]. Bioinformatic analysis of 1,037 fungal genomes from the Ascomycota, Basidiomycota, and non-Dikarya revealed that the number of BGCs per genome significantly varies across fungal genomes.[9] In the Ascomycota phylum Pezizomycotina genomes harbor on average 40 SM BGCs (25% of the genomes within this class possess >60 BGCs), however, this number is significantly lower in non-Dikarya (~15 BGCs), in Basidiomycota (<10 BGCs) or non-Pezizomycotina Ascomycota genomes (~5 BGCs)[9]. Because of the richness and diversity of SM BGCs contained within their genomes, Pezizomycotina fungi are by far the most studied taxon in the field of SM discovery. Unfortunately, most of the BGCs encoded in their genomes are transcriptionally silent under laboratory cultivation conditions.[10]

Fungal SM BGCs can be activated via manipulation of cultivation conditions or by genetic modifications. Using different cultivation conditions or co-cultivation with other organisms[11,12] has led to successful examples of BGC activation, as we further discuss in a later section. Replacement of the promoter driving the expression of local or global transcriptional regulators is a commonly used genome editing strategy for transcriptional activation, e.g. overexpression of transcriptional activators or knock-outs of transcriptional repressors, as well as manipulation of epigenetic modulators, which function as global chromatin regulators.[13] Traditional metabolic engineering methods combined with the implementation of the "clustered regularly interspaced short palindromic repeats" (CRISPR) technologies[14] further accelerated strain construction and enabled more complex and sophisticated genetic engineering of filamentous fungi.[15] BGCs can be transcriptionally upregulated by conventional genome editing approaches, but thanks to the latest developments in synthetic biology, new attractive genetic tools have become available: synthetic transcription factors (STFs), artificial transcription units, fungal shuttle vectors, and various enhanced platform strains for heterologous expression. In the following sections

we present recently developed tools and discuss how they compare to each other and to conventional metabolic engineering approaches.

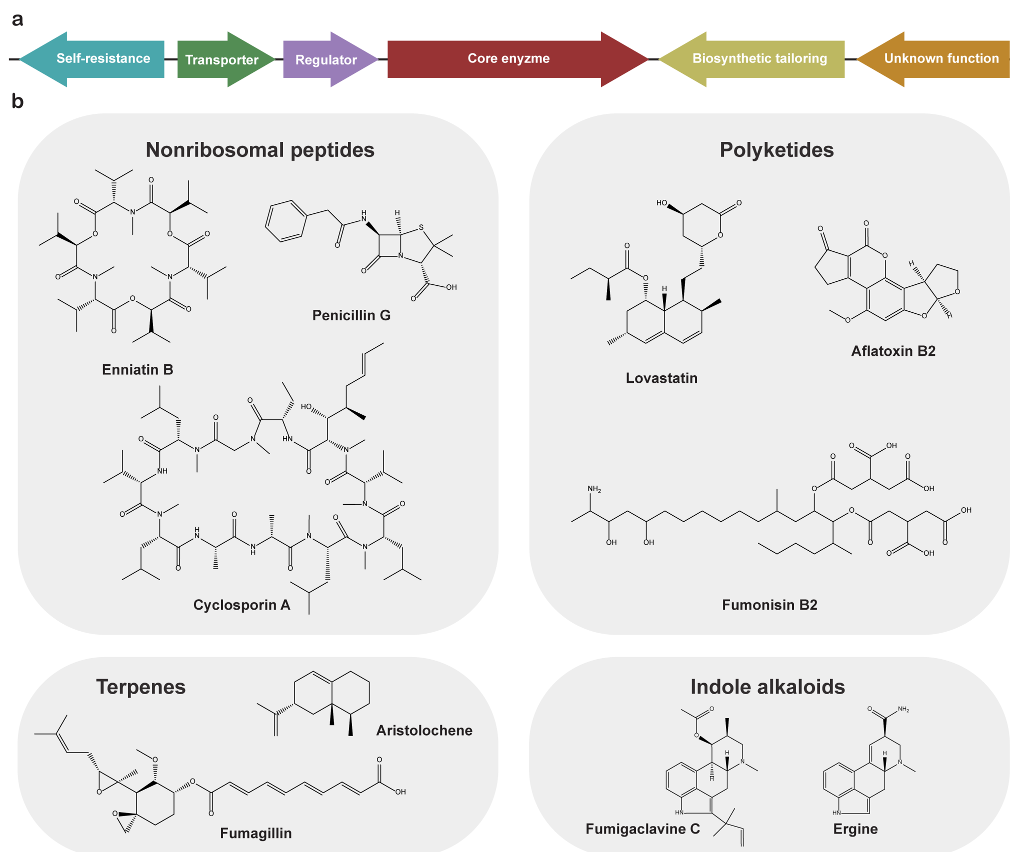

Figure 1. Schematic representation of a fungal biosynthetic gene cluster (BGCs) (a) and structurally different, representative members of nonribosomal peptides synthetase (NRPS), and polyketides synthase (PKS), terpenes synthase produced secondary metabolites and indole alkaloids from fungi (b).

Fungal secondary metabolite biosynthetic gene clusters

SMs are low molecular weight, structurally heterogeneous compounds–synthesized by bacteria, fungi, and plants–which are not directly involved in the normal growth, development, or reproduction of the organism. SMs are synthesized from metabolic intermediates from primary metabolism. The produced SMs commonly provide a biological advantage to their producers, to thrive and survive in their environment, for instance in supporting the competition against other organisms (toxins and antimicrobials) and in the protection against harsh environments (pigments and iron-chelating siderophores), but SMs are also used for chemical signaling.[16] Although many SMs have no known function, these compounds probably fulfill a role in complex communication networks in ecosystems, but so far it is just a human interpretation with limited experimental evidence.

The core skeleton of fungal SMs is produced by dedicated biosynthetic enzymes that belong to a few distinct families: nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS), terpene synthases (TPS), dimethylallyltryptophan synthases (DMATS), or combinations thereof.[10] NRPS and PKS are complex multi-modular megaenzymes that utilize a variety of amino acids and acyl-CoA monomers as substrates, respectively. TPS and DMATS are generally smaller and use a more limited set of substrates: the former use intermediates of the mevalonate pathway (IPP and DMAPP) as starter units for terpene synthesis[10]; the latter utilize DMAPP to prenylate the amino acid tryptophan or other aromatic substrates.[17] In all cases, the core scaffold is generally further modified by tailoring enzymes (oxidases, reductases, methyltransferases, cytochrome P450 monooxygenases, and others) whose genes are often found in the same BGC, ensuring a broad chemical diversity of the products (Figure 1b). Furthermore, these clusters frequently contain genes encoding transporters and regulatory proteins (Figure 1 a). The size of a BGCs can span from a few kb to ~100 kb incorporating as little as only two genes (valactamide BGC)[18] or up to ~27 genes (aflatoxin BGC).[19] Given that they are the most abundant in filamentous fungi—particularly in more commonly studied

members of Pezyzomycotina—we will mainly discuss NRPS- and PKS-encoding BGCs in the remainder of this section.

NRPS enzymes synthesize a broad class of small peptides, typically 2-50 monomers from a wide variety of amino acids and their derivatives, as well as fatty acids and alpha hydroxy acids.[20] These enzymes have a modular structure, where each module is responsible for the activation and coupling of a monomer to a growing peptide chain. A minimal NRPS module consists of an adenylation (A), condensation (C), and thiolation (T) domain (also called peptide-carrier protein). The A domain recognizes the monomer substrate and activates it as an (amino) acyl-AMP conjugate, which is subsequently transferred to the T domain via a transesterification reaction. The activated substrates/intermediates are then transported to the C domain, which is responsible for the formation of the peptide bond. Eventually, the final product is released by a terminal thioesterase domain.[20,21]

Polyketides represent the most abundant group of SMs. PKS enzymes utilize activated short-chain organic acids derived from primary metabolism, such as acetyl-, malonyl- or methylmalonyl-coenzyme A, for the biosynthesis of polyketides. The basic set of domains consists of an acyl carrier protein (ACP), a β-ketoacyl synthase (KS), and an acyltransferase domain (AT). The AT domain selects and loads both starter and extender monomers, while the KS domain catalyzes C-C bond formation between two adjacent substrates/intermediates. The ACP domain is responsible for storing and shuttling monomer substrates and synthesized intermediates during the elongation process. PKS enzymes are extremely diverse, many contain optional domains that introduce further chemical modifications, generating an incredible variety of products.[21,22]

Molecules can be also constructed from hybrid NRPS-PKS assembly lines leading to mixed NRP-PK products, such as the bacterial bleomycin, rapamycin, epothilones, or the fungal fusarin C, pseurotin A, tenellin, and cytochalasin E. [21,23] NRP-PK hybrids can be synthesized by proteins containing domains and modules from both PKSs and NRPSs organized in the same polypeptide chain (tethered), but these enzymes can also be formed from individually expressed proteins

in the BGC (non-tethered).[24] In these hybrid systems, the different subunits need to communicate efficiently to coordinate the transport of substrates and intermediates across the hybrid system, and have to perform either C-C or C-N bond elongations at the corresponding PKS/NRPS interfaces.[21]

BGCs encoding NRPS and PKS clusters can readily be predicted and identified from genomic data by advanced bioinformatics algorithms, for example using the conserved domains of the core enzymes. Such tools are the "Secondary Metabolite Unique Regions Finder" (SMURF)[25] or the "Antibiotics and Secondary Metabolite Analysis SHell" (antiSMASH)[5]. AntiSMASH is continuously updated since its release in 2011, incorporating several newly developed algorithms, e.g. searching for shared transcription factor (TF) binding sites in the promoter sequences ("Cluster Assignment by Islands of Sites" (CASSIS)[26]). Cross-referencing with public databases further aids the identification of uncharacterized BGCs. One example is the MIBiG (Minimum Information about a Biosynthetic Gene cluster) repository, which contains curated information in a unified format listing the BGC annotations and their molecular products.[6] As these algorithms accept annotated DNA sequences as input, cluster predictions can be further advanced taking into account transcriptome data of the predicted BGC, assuming that the cluster can be brought to a transcriptionally activated state. Algorithms such as MIDDAS-M (motif-independent *de novo* detection algorithm for SM BGCs) aim to combine genomic data and transcriptomic data to predict coordinately regulated genes including fungal BGCs.[27] As homologues of clusters with known compounds can be easily identified, the use of databanks and algorithms can reduce the re-discovery rates or yield predictive information regarding the targeted BGCs. The discovery of a huge number of transcriptionally silent BGCs through bioinformatics fueled the interest in genome mining, and the interrogation of these unknown clusters by experimental identification.[28,29]

Regulation and transcriptional activation of BGCs

SM production is often regulated by a stimulus, and without it, the product of the BGC is not synthesized. When no known metabolite is connected to a BGC,

the cluster is called "cryptic" or "orphan". In most cases, BGCs react to various environmental stimuli, but often the connection between regulators and the stimuli is unknown. Under laboratory conditions, native environmental signals may not be present, rendering BGCs transcriptionally silent. Since cryptic BGCs appear to be silent under laboratory conditions[10], alternative strategies need to be employed to awaken these clusters and explore their biosynthetic potential.

Conventional methods for transcriptional activation of genes or even entire BGCs have been rapidly implemented in fungal biotechnology[13,30]. One of the strategies concerns the OSMAC (One Strain MAny Compounds) approach, which assumes that one strain is capable to produce numerous compounds, but different environmental or cultivation conditions regulate what subset of BGCs are activated.[11] Indeed, modifications for cultivation parameters such as temperature, salinity, aeration and others, showed that *Aspergillus ochraceus* is capable of producing 15 compounds in addition to the previously known aspinonene.[11] Co-culturing can also result in transcriptional activation of BGCs due to inter-species crosstalk[12,31,32]: co-cultivating *A. nidulans* with the soil-dwelling bacterium *Streptomyces rapamycinicus* resulted in the production of orsellinic acid.[33] Co-cultivation of *A. fumigatus* with the same bacterium resulted in the activation of the fumicycline BGC, which involved epigenetic regulation changes induced by the bacterium.[34]

Global regulators

Around half of the fungal BGCs do not harbor in-cluster regulators, and are only regulated by global transcription regulatory mechanisms.[10] Global transcriptional regulators respond to environmental stimuli by coordinated up- or downregulating of required gene sets, and the corresponding TFs to these signals have been identified in several cases: CreA responds to carbon levels; the velvet complex to light; AreA to nitrogen concentration; PacC to pH levels; and the CCAAT-binding complex to iron concentration.[35] These regulators act genome-wide on numerous genes, controlling morphological development, primary metabolism as well as SM production. Both overexpression (e.g. LaeA transcriptional activator of

secondary metabolism)[36] and deletion of master regulators (e.g: McrA repressor protein)[37] resulted in transcriptional activation of BGCs.

Chromatin-mediated regulation

Alterations in the structure of chromatin can result in global transcriptional regulatory effects.[38] Histones are critical proteins responsible for the tight packing of DNA in the nucleus, creating the chromatin. Histones can undergo numerous modifications (acetylation, methylation, phosphorylation, uniquitination, sumoylation, and neddylation), and this can create less- or more accessible DNA segments.[39] Histone deacetylation results in a more closed chromatin structure causing transcriptional repression of affected genes. In contrast, histone acetylation can result in more accessible chromatin for regulator proteins and the transcription machinery, causing transcriptional activation. The structure of chromatin can be manipulated by using chemical agents or genetic modifications to achieve transcriptional regulation of genes of interest. For instance, chemical histone deacetylase (HDAC) inhibitors (e.g. suberoylanilide hydroxamic acid, trichostatin, and sodium butyrate) can be supplemented to the cultivation media to prevent histone deacetylation.[39] Such epigenetic perturbation may lead to a 100-fold up- and downregulation of genes that are spread over the genome.[40] Epigenome editing for transcriptional activation is also possible by genetic engineering of the regulation of the expression of histone acetyltransferase (HAT) or HDAC genes. In *A. nidulans*, downregulation of the gene *rpdA* that encodes a HDAC enzyme yielded similar results as observed with chemical HDAC inhibitors.[40] Deletion of the *hdaA* (histone deacetylase A) in *A. nidulans* resulted in increased penicillin and sterigmatocystin production[41]. In *P. rubens* (previously identified as *P. chrysogenum*[42]), deletion of a *hdaA* homolog positively affected the production of sorbicillins and roquefortine/meleagrin[43], and significantly downregulated the BGCs responsible for chrysogine and dihydroxynaptelene-melanin production[44].

Cluster specific regulators

Around half of the predicted BGCs harbor genes encoding TFs, which are often transcriptional activators of the complete cluster[45]. These regulators bind to the corresponding recognition sequence in the promoters of the genes in the BGC. As promoter replacement is a good strategy to override the native regulation of a transcriptionally silent gene, replacing the promoter of the in-cluster TF can result in cluster-specific activation. Although overexpression of cluster specific TFs has led to the production of aspyridones, asperfuranone and emodin derivatives in several Aspergilli[45–47], a systematic promoter replacement approach in *A. nidulans* showed that only 3 out of 17 overexpressed cluster specific TFs effectively led to the production of an obtainable amount of SMs[48]. Although inducers and protein-protein interactions affect the activity of the TF, it is currently unknown what other mechanism(s) are required for complete BGC activation alongside the overexpression of an in-cluster TF. Cross-talk between different cluster specific TFs have also been described in *A. nidulans*, as the overexpression of the ScpR TF (from the fellutamide BGC) caused upregulation of the in-cluster *inpA* and *inpB* NRPS genes, as well as the asperfuranone BGC, located on a different chromosome[49]. Since the AflR (aflatoxin transcriptional activator) recognition sequence can be found in most of the promoters in the sterigmatocystin and aflatoxin cluster, this TF was shown to be able to regulate positively both BGCs, as well as some genes outside of these BGCs[50,51].

In-cluster SM BGC transcriptional repressors with DNA-binding capacity have so far not been discovered in filamentous fungi. Rather, repressor proteins interacting with transcriptional activators is a more common mechanism. For example, the primary metabolism BGC responsible for the quinic acid degradation in *N. crassa* is controlled by a transcriptional activator/repressor regulator pair (qa-1F/qa-1S)[52]. Similarly, in *A. niger* the repression of galacturonic acid utilization pathway is modulated by a regulator pair (Gaar/GaaX)[53]. It is believed, that these in-cluster repressors are responsible for keeping the positive transcriptional regulator inactive in the absence of an inducer[52,53]. The sorbicillin SM BGC in *P. rubens* harbors an activator/repressor pair as well, and the metabolites of

the cluster are acting as autoinducers for the pathway[54]. Overexpression of the transcriptional repressor (*sorR2*) results in transcriptional suppression, while deletion of *sorR2* results in early-stage transcriptional activation of the sorbicillin BGC, but with hardly any sorbicillin production[54]. Although promoter replacement, gene deletion or complete BGC refactoring in the native host leads to direct transcriptional activation of the gene of interest, but these methods require editing the genome of the fungus.

Fungal genome editing

Precise and flexible genome editing is key for efficient engineering of the fungal host. Targeted gene manipulation in wild type filamentous fungal species is challenging due to the relatively low rates of homologous recombination (HR) and high rate of random integration of the transformed DNA. Targeting efficiency to the desired location is relatively low in *Aspergillus* and *Penicillium* species (0.1 - 5.0 %)[55,56], and it differs by the organism and targeted locus. The fungal homologs of the human *ku70/ku80* genes encode a protein complex functioning in the non-homologous end-joining (NHEJ) DNA repair pathway, which favors random integration of the transformed donor DNA. Deletion of either of these genes is highly advantageous for fungal strains that are employed for precise genome editing and HR-mediated DNA delivery. Inactivation of either of the fungal homolog of the human *ku70/ku80* genes drastically decreases or eliminates the functionality of the NHEJ DNA repair pathway and highly increases the efficiency of targeted DNA delivery through HR.[56–59] With the advent of CRISPR-based tools the genome editing efficiency was further increased, in some filamentous fungal strains reaching more than 90%.[60–63]

A commonly used alternative to engineer fungal hosts is the *Agrobacterium*-mediated transformation, particularly employed when little or no genetic tools are available for that host. *A. tumefacians* is a gram-negative plant pathogen that has been shown to be capable of transferring its transfer-DNA (T-DNA, used by the bacteria to infect plants) into the genome of several filamentous fungi. This made it possible to achieve successful DNA delivery in fungal hosts that

cannot be transformed with traditional methods[64,65]. The system is commonly used for random integration of one single copy of a gene of interest, but it can also be used for targeted genome editing in NHEJ-deficient hosts via homologous recombination using long homologous flanking sequences (>1000 bp).[65]

Before the global application of CRISPR-based genetic tools, zinc finger nucleases (ZFNs) and transcription activator-like effector (TALE) nucleases were established for locus-specific genome engineering applications. Engineering the DNA binding domain (DBD) of these nucleases allows targeting specific genomic loci. ZFNs are artificial restriction enzymes and are typically generated by fusing DBSs with the FokI nuclease domain. DNA targeting is provided by fusing together three to six DNA-binding zinc-finger proteins, each of which is capable to recognize a specific 3 bp DNA sequence. Although ZFNs are relatively small proteins, which are easy to deliver to the host, their targeting efficiency is rather weak and the relatively high levels of off-target effects may lead to cytotoxicity.[66] The next generation of targeted DNA editing was the discovery of the transcription activator-like effector nuclease (TALEN) elements, which are acting as TFs in the species of *Xanthomonas*. The DBD builds up from 33-34 amino acid long tandem repeats, which determines the targeted DNA sequence. These repeats can be altered to recognize one specific nucleotide and by combining these repeats in sequential order, the protein can be targeted to any DNA sequence (preceded by a thymine or cytosine base). Direct fusion of the TALE DBD and with restriction endonuclease (FokI) domain created guidable TALENs, meanwhile fusions to transcription activation domains (ADs) to created STFs for targeted transcriptional regulation (TALE-TFs).[67]

CRISPR-mediated genome editing

The CRISPR systems and their CRISPR-associated (Cas) proteins have recently been repurposed for transcriptional gene regulation in eukaryotes[68,69], where they can be utilized as components of STFs. Native Cas proteins provide a self-defense mechanism against bacteriophage virus infection in prokaryotes. When these organisms encounter the virus for the first time, they embed small

viral sequences in their genome, which are later transcribed into small RNAs molecules. These RNAs form complexes with the Cas proteins, which are now able to recognize and cleave the complementary nucleic acid sequence in the viral genome at any next infection event, effectively eliminating the intruder.[70] Cas proteins commonly cleave double or single stranded DNA, but RNA-cleaving Cas proteins have also been identified.[71] Repurposing and utilizing these systems for targeted genome editing has revolutionized precise genome engineering in various organisms. In these two component CRISPR systems, the Cas protein is guided by a CRISPR RNA (crRNA) to a target specific locus for nucleic acid cleavage. For the commonly used Cas9 systems, specificity is delivered on a single guide RNA (sgRNA) complex that encodes both the short trans-activating CRISPR RNA (tracrRNA) and crRNA transcripts. The tracrRNA forms the stem loops that anchor the endonuclease protein while the crRNA is the actual targeting sequence. In contrast, the other commonly applied Cas12a (Cpf1) nuclease is capable to processes its own crRNAs from pre-crRNA, and does not need a tracrRNA.[72]

With the Cas9 system, the genomic locus is targeted by a sequence-specific 17-20 nucleotide crRNA which is complementary to its genomic target (protospacer), that must be followed by a protospacer adjacent motif (PAM). This PAM sequence is recognized at the DNA level by the protein and is unique for different Cas proteins. These unique PAM sequences limit the number of sequences that one can target since they show minimal flexibility for different nucleotides: For example, the PAM sequence recognized by the commonly used *Streptococcus pyogenes* Cas9 (SpCas9) is 5′-NGG-3′ (to a lesser extent non-canonical NAG and NGA are also recognized, where N is any nucleotide) located downstream the protospacer, meanwhile the Cas12a nuclease recognizes 5′-TTTV-3′ (where V can be G, C or A) sequences located upstream of a typically 20-24 nucleotide long protospacer.[73] Careful design of the crRNA is therefore essential to avoid off-target CRISPR effects, as the nuclease complex is capable to bind to highly similar sequences[74], which represents another limitation of this system.

In recent years, highly efficient CRISPR-based genome editing tools have been developed and established for several organisms, such as bacteria, yeast, and human cells.[75] CRISPR/Cas9-based genome editing in filamentous fungi has been established for several organisms including *A. fumigatus*, *A. oryzae*, *Neurospora crassa*, *Pyricularia oryzae*, *Trichoderma reesei*, *Ustilago maydis*, and *P. rubens.*[15] CRISPR elements can be delivered as ribonucleoproteins preassembled *in vitro*, or as genetic elements that are expressed by the host. The AMA1 (autonomous maintenance in *Aspergillus*) sequence from *A. nidulans* supports autonomous vector replication in several filamentous fungal species[76,77], and thus vectors encoding this sequence have been extensively used for gene delivery and expression purposes, as well as delivering CRISPR components.[60,78–81] Single vector-based CRISPR/Cas9 genome editing systems have been previously developed for several filamentous fungal species.[15] Recently, a similar system was developed based on the nuclease Cas12a (Cpf1) for Aspergilli.[82]

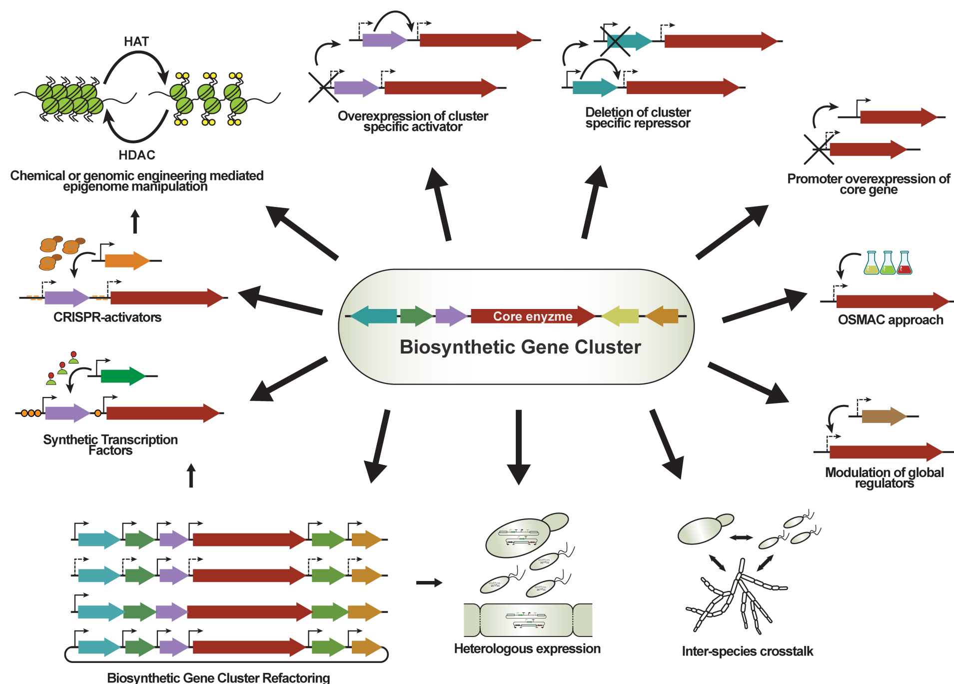

Figure 2. Strategies for transcriptional activation for fungal biosynthetic gene clusters. Dashed arrows indicate native, solid arrows indicate engineered (strong or inducible) promoters.

Fungal Synthetic Biology Tools

Synthetic biology has revolutionized metabolic engineering with tools–created by repurposing or redesigning biological systems found in nature–enabling the exploitation of industrial microorganisms at whole new levels. Since synthetic biology strives to engineer highly predictable and controllable genetic systems, genetic circuits are often constructed in a standardized and preferably modular fashion. The modularity of various DNA parts encoding genetic elements allows rapid assembly of novel, more predictable genetic circuits, like logic gates and genetic switches. Inducible or synthetic transcriptional regulators (activators and repressors) can be used to enable fine-tuning of gene expression or controlling entire pathways. Synthetic biology-based tools have been established in several model bacterial and eukaryotic systems, and recently also in filamentous fungi where they are still relatively underdeveloped compared to more common hosts.

Modular Assemblies

The Design-Build-Test-Learn (DBTL) cycle of synthetic biology represents a systematic and efficient workflow for the optimization of biological systems for specific functionalities (e.g. strain improvement). Complex genetic systems can be constructed in a modular manner with desired features–synthetic regulatory tools and rewired expression of biosynthetic pathways–enabling an affordable genetic engineering of biological systems. Synthetic transcription units can be rapidly assembled by cloning methods supporting the assembly of multiple DNA fragments (e.g. Gibson Assembly[83] or USER Cloning[84]), or high-throughput, modular cloning methods, such as Golden Gate cloning-based[85] Modular Cloning[86], and GoldenBraid[87] assemblies. With these methods, genetic parts (promoters, coding sequences, and terminators) can be arranged into transcription units, where the building blocks are interchangeable within the same synthetic biology language, but their order and orientation are commonly predetermined. Collections of such DNA building blocks (toolkits) have been established for bacteria[88], yeasts[89,90], plants[91], mammalian host cell lines[92] and also for filamentous fungi[93–96]. These toolkits can provide backbone vectors to facilitate modular assembly and

fungal delivery, or pre-assembled vectors containing various genetic elements, suitable for generic applications or specific needs. The deposition of such toolkits containing ready-to-use, established DNA parts or modules, highly accelerates the biological DBTL cycle for synthetic biology applications. A major repository for genetic parts is Addgene, a free online database that facilitates the exchange of genetic material between laboratories around the world.

Artificial promoters and Synthetic Transcription Factors

Eukaryotic promoters are complex DNA structures responsible for recruiting transcriptional regulatory elements (transcriptional regulators). The simplest functioning unit of the promoter—often called minimal or core promoter (CP)—is incorporating the transcription start site (TSS) and is required to initiate transcription of the gene of interest. CPs contain specific DNA elements that the RNA polymerase II requires to initiate transcription.[97] In eukaryotes, CP sequences are highly diverse: many motifs can be present such as the TATA, CCAAT boxes, the B recognition element, and the initiator element.[98] Several regulatory TF-binding sequences are located upstream of the CP sequence, where they recruit transcriptional activator or repressor proteins, hence, modulating the transcription of the gene. The length of CPs is not well defined in filamentous fungi, but these sequences are located roughly 140-200 bp upstream of the starting codon.[99,100] The precise identification of upstream regulatory DNA sequences and CPs is essential for the engineering of functional synthetic promoters.

Synthetic gene expression systems can be used for the production of metabolites or proteins of interest, and have been established in numerous filamentous fungal species.[99–101] Such orthogonal systems do not rely on the regulatory system of the host, but instead depend on hybrid or synthetic TFs, composed of different DNA-bindings (DBDs) and transcriptional effector domains, and on synthetic promoters. DBDs target and bind to unique upstream activating sequences (UASs) in the promoter region, effectively regulating gene expression. If the TFs are inducible and/or repressible upon the addition of small molecules, these systems can be used as genetic switches as well. STFs have

been repurposed from different prokaryotic, eukaryotic, or viral transcriptional regulators and have been shown to be functional in several hosts including yeast and filamentous fungi. Using such synthetic transcriptional regulators, activation or repression of genes can be achieved in a controlled manner. Transcription can be fine-tuned for each gene individually by changing different elements of the system. Synthetic promoters created by fusing specific UAS and CPs, or by integrating UAS elements into native promoters, can be used to rewire the native transcriptional regulation system of the genes of interest. Synthetic promoters bring the promise of a pre-defined, fine-tuned, and metabolism-independent expression for multiple individual genes. Synthetic promoters in combination with an inducer-dependent STF can allow further tuning of gene expression in a gene dosage and/or inducer concentration dependent manner.[102] Such refactoring would allow the overexpression of entire BGCs bypassing the need for established strong promoters for each gene of the cluster, since the number of such promoters is limited for filamentous fungal hosts. Functional STFs have been successfully introduced in Aspergilli[99,101,103,104], *P. rubens*[100], *T. reesei*[105], and *Ustilago maydis*[106].

Many STFs have been constructed to regulate genes in primary metabolism.[107–111] Fusing the DNA-binding domain of the CreA/Cre1 (carbon catabolite repression) transcription factor to the complete Xyr1 transcription factor (Xylanase Regulator 1) resulted in enhanced cellulase production in a CreA/Cre1 deficient *T. reesei* strain grown on glucose.[112] Fusing the DBD of the Xyr1 with the regulator domain of Ypr2 (transcriptional activator of the sorbicillinoid SM BGC) resulted in high expression of xylanases and cellulases in *T. reesei* nearly independently from the carbon source used.[105] Replacing the regulatory domain of a weak in-cluster transcriptional TF with a highly active activator domain (AD) can lead to activation of target SM BGCs, without the integration of additional synthetic promoter elements. When the DNA-binding domain of the transcriptional activator (AlnR) from the asperlin BGC was fused to the regulatory domain of the transcriptionally highly active asperfuranone TF (AfoA), it led to the production of asperlins in *A. nidulans.*[104] In these works, the

DBD in the STF retained its capability to bind to its native operator sequences in the promoters, while the newly fused activator domain provided transcriptional activation of the genes.

Although STFs (also called altered, artificial, or hybrid TFs) have been studied for more than 30 years (often using the Gal4 TF as a model from *Saccharomyces cerevisiae*)[113–115], there is limited information about how these domain fusions should be engineered to avoid creating nonfunctional STFs. Cluster-specific TFs commonly consist of a $Zn(II)_2Cys_6$ (C6 zinc) DBD and a transcriptional regulator domain. DBDs often contain at least one structural motif that recognizes and bind to double- or single-stranded DNA sequences. Generally, DBDs can be further divided into sub regions: the zinc finger, the linker region, and a coiled-coil element. Numerous Gal4-family TFs contain a coiled coil between the linker and the regulator domain, which is possibly responsible for mediating protein-protein interactions or homodimer formation before binding to DNA.[113] While structural changes in the zinc-finger motif, the linker region or the coiled-coil regions negatively affect the functionality of the TF, the regions between these coiled-coil sequences and the regulator domains are often non-essential for retaining activity.[114–116]

The tetracycline-inducible (TET) expression system has been originally developed for mammalian cells[117], and later adopted for other eukaryotic systems, as well as for Aspergilli and *U. maydis*[101,102,106]. Within the endogenous tetracycline-resistance system in Gram-negative bacteria, the TetR transcriptional repressor represses the expression of the tetracycline transporter gene (TetA) by binding to the TetO (or "tetracycline response element" TRE) operator sequences in the promoter. In the presence of the antibiotic tetracycline, TetR will bind the compound and be released from TetO, enabling expression of the transporter gene which eventually provides self-resistance[118]. This repressor was engineered into an activator in the Tetracycline-Off (Tet-Off) system, where a tetracycline-controlled STF, the tTA (TetR-VP16 fusion) provides inducible repression. Transcriptional repression can be achieved by feeding tetracycline (or its synthetic derivative doxycycline) to the medium, which binds to the synthetic activator (tTA), thus

preventing binding to the TetO sequences and the expression of the gene of interest. Several copies of the TetO sequences are inserted upstream of a weak or transcriptionally silent minimal (core) promoter for transcriptional regulation of the gene of interest. As tetracycline and doxycycline have relatively short half-lives, these chemicals need to be added to the medium repeatedly to maintain transcriptional repression, and in their absence transcriptional activation occurs, as the tTA binds to the TetO sequences.

Using the further engineered Tetracycline-On (Tet-On) system, gene activation can be achieved in a concentration-dependent manner by feeding the inducer and using the reverse tetracycline-controlled transcriptional activator (rtTA, mutated TetR-VP16 fusion) as the STF. In the presence of tetracycline (or doxycycline), the affinity of this rtTA STF towards the TetO sequences increases, therefore enhancing the transcription of the gene of interest downstream. Unfortunately, the rtTA retains some binding affinity to its TetO sequences in the absence of the inducer, leading to leaky transcription. Thus, an advanced version of rtTA (rtTA2^{S}-M2, TetR-3xVP16) was designed, showing increased specificity, stability, and inducibility using doxycycline without leaky expression.[119] This Tet-On system was applied with *A. fumigatus* for inducible expression of the gene of interest using seven copies of the TetO sequence upstream a short 175 bp CP sequence of the commonly used *gpdA* promoter.[101] The system was established in *A. niger* using fluorescent reporters, and was applied for the production of fructose-6-phosphate amidotransferase[103] and biologically active fungal cyclodepsipeptides (Enniatin B, Beauvericin, Bassianolide) on a grams-per-liter scale[120].

The bacterial Bm3R1-based STF (Bm3R1-DBD-VP16) was shown to be functional for transcriptional activation in yeasts as well as in *A. niger* and *T. reesei.*[99] This STF was delivered to different fungal hosts harboring several copies of the BS-UAS, enhancing the transcription capacity of various, native and non-native CPs to control gene expression in fungi. These results showed that, although CPs function differently among hosts, universally functional CPs which operate both in filamentous fungi and yeast hosts can be designed. Some

of these synthetic promoters even performed better than commonly used native "strong" promoters. As the native TFs have no known inducers, controlling or inducing the transcription in this system is not established.

The transcriptional activator and repressor of the quinic acid metabolism from *Neurospora crassa*[121] have been implemented as a binary expression system for *Drosophila melanogaster* and mammalian cell lines, known as the "Q-system".[122] This system has controllable features as in the native host the repression of qa-1F by the qa-1S transcriptional repressor can be relieved by feeding with quinic acid, resulting in inducible activation. In the earliest example of an engineered Q-system, a STF was constructed by fusing the DBD of the qa-1F transcriptional activator to the GAL4 AD. This DBD binds to its corresponding recognition sequences upstream of the targeted promoter (called QARE QA response element or QUAS Q-System UAS).[121,122] The Q-system was later also adapted and established for mammalian cells, *Caenorhabditis elegans*, zebrafish, and malaria mosquitos.[123] Based on the Q-system transcriptional activator (qa-1F), a STF using the VP16 AD (qa-1F-DBD-VP16-GFP) has been constructed in *P. rubens*, where the strength of the Q-system STF device showed scalability by using different CPs, by increasing the expression levels of the STF or the number of UAS elements (1, 5 or 11) upstream of the CP.[100] The system was capable to produce expression levels ranging from hardly detectable to a level similar to that of highly expressed native genes. These synthetic expression devices were validated using fluorescent reporters while the application potential was confirmed by synthetically controlling the expression of the penicillin BGC. The development of such a system further increased the number of genetic regulation tools available for filamentous fungi.

CRISPR-based transcriptional regulation

Mutations in the nickase domain(s) of the CRISPR protein eliminate its nuclease activity while retaining the capability of the protein to bind to the DNA. Such "nuclease-dead" CRISPR proteins (dCas) are engineered from Cas9 by introducing point-mutations in the RuvC and HNH nuclease domains (in dCas9m2 from *S.*

pyogenes these are the D10A and H840A, respectively). Similarly, point mutations are introduced in the RuvC-like domain of Cas12a to generate its corresponding dCas variant (E993A in dCas12a from *Acidaminococcus sp.*).[124] These proteins can be fused to ADs and used as STFs (CRISPRa, activation), thereby recruiting a transcriptional regulator to the promoter of the gene of interest. Since dCas proteins can still bind tightly to their target sequences, they can be guided to regions upstream of a gene of interest where they form a "road-block" for the transcriptional machinery, resulting in transcriptional repression (CRISPRi, interference). Taking advantage of the guidable DNA-binding capability, these dCas proteins can be applied for various other applications depending on the delivered modulator, e.g. targeted DNA modifications (e.g. methylation), transcriptional regulation, fluorescent imaging can be achieved.[73] Inactivated Cas proteins can be used to deliver transcriptional regulator domains to the promoter of the gene of interest by direct fusion of regulatory domains, or repetitive peptide epitopes that recruit multiple copies of antibody-fused regulators (SunTag), or by using MS2 RNA stem-loops in the sequence of the tracrRNA to recruit MS2-tagged regulators (Synergistic Activation Mediator "SAM" system).[125,126]

The protospacer sequence is crucial in CRISPRi/CRISPRa applications for targeted repression and activation, respectively. CRISPRi has been successfully adapted to several bacterial and eukaryotic hosts for targeted gene repression.[69,127–134] Targeting in close distance to the TSS of the gene of interest with this system leads to successful downregulation, presumably by blocking transcriptional initiation or elongation. In prokaryotic CRISPRi applications, the bare dCas9 without any fused regulator domain is already capable of achieving significant repression. It is believed that the binding of dCas9 can hinder the binding of positive enhancers or the mediator complex for transcriptional elongation. In eukaryotes, the levels of repression achieved by using dCas9 alone are low, but can be enhanced by fusing repressor domains such as KRAB (Krüppel associated box) or Mxi1 (a histone deacetylation mediator).[128] The efficiency of CRISPRi-based repression differs depending on several factors including the type of fused effector, off- and on-target effects of the CRISPR protein, the distance

of the protospacer from the TSS, and the chromatin state of the target genomic region.[135] Presumably, the native transcription levels of the target genes and the presence of regulatory protein binding sequences in close proximity of the CRISPR complex also affect the degree of repression. In mammalian cell lines, the dCas9-KRAB fusion provides repression when targeted in the range of -50 to +300 bp relative to the TSS of a gene, with the highest efficiency of ~100-fold repression in the -50 to +100 bp region.[127] In *S. cerevisiae*, the dCas9-Mxi1 fusion resulted in a maximal ~10-fold repression when targeted to the -200 to +1 region relative to the TSS, but this reduced efficiency could be explained by the mode of repression employed by Mxi1, which mediates DNA deacetylation.[135] These experiments also highlight how nucleosome occupancy and chromatin accessibility can affect crRNA efficiency. The level of repression can be further increased by deploying multiple sgRNAs in combination with the Cas9 systems[134] which can be achieved by using sgRNA-arrays in combination with self-cleaving sequences (Hammerhead and HDV ribozymes and tRNA)[136,137] or exogenous nucleases and their cleavage factor recognition sequences (Csy4 nuclease)[138].

CRISPR-based transcriptional activation systems commonly use the VP16 AD or variants thereof where the VP16 is arranged in tandem repeats (VP64, VP160). This regulatory domain originates from herpes simplex virus, but it was shown to function in various organisms.[99–101,117,139] VP64 was also combined with two other potent transcriptional activators to generate the VPR (VP64-p65-Rta) tripartite activator domain, which has been shown to be superior compared to other activator domains tested in human, mouse, and fly cell lines as well as in the yeast *S. cerevisiae.*[140] The Cas-VPR fusion system has been successfully adopted for filamentous fungi, and established for *A. nidulans*[141], and *P. rubens*[142]. In *A. nidulans*, the dCas9-VPR and dCas12a-VPR activators were expressed from an episomal vector and were guided to the transcriptionally silent *elcA* promoter of the PKS gene of the elsinochrome BGC from *Parastagonospora nodorum*, which was fused to an mCherry fluorescent reporter gene. After transcriptional activation of *elcA* was validated using fluorescence microscopy, the system was used to overexpress individual genes of the native microperfuranone BGC in

A. nidulans, which resulted in enhanced production of microperfuranone and the identification of dehydromicroperfuranone[141]. In *P. rubens,* a vector-based dCas9-VPR system was used to activate the transcriptionally silent, native *P. rubens* macrophorin BGC by activating the promoter of the transcriptional activator of the cluster[142]. This CRISPR activator system was validated using a transcriptionally silent CP[100] driving a DsRed fluorescent reporter[142]. Cas12a is natively able to process its own crRNAs from an array of pre-crRNAs, while Cas9 requires additional engineering for the delivery of multiple crRNAs (e.g. individual sgRNA transcription units, self-cleaving ribozyme sequences, or Csy4 endoribonuclease cleaving)[138]. Since targeting the same promoter with multiple crRNAs shows synergistic effects in various eukaryotic CRISPRa applications[143], dCas12a systems are superior compared to dCas9 for gene regulation purposes. In mammalian cell lines, CRISPRa seems to be the most effective in the range of 400 to 50 bp upstream of the TSS.[127] Since the genes of cryptic BGCs in filamentous fungi are often transcriptionally silent, the TSSs are not known. In this case, crRNAs can be designed to target regions close to the predicted TSS or to the start codon of the gene of interest. Both in *A. nidulans* and *P. rubens*, this approach was successfully used to achieve transcriptional activation using individual sgRNAs guiding the dCas9-VPR activator to 162-190 bp (P*elcA*) or 106-170 bp (P*penDE*) and 68-73 bp (P*macR*) region upstream of the start codon, respectively.[141,142] Next to the general rules to identify CRISPR protospacer candidates (selecting predicted high on-target and low off-target binding efficiency, and avoiding strong secondary RNA structures), regulatory DNA elements in the targeted promoter, as well as local chromatin organization should be considered when designing crRNA sequences.

When designing CRISPRa strategies, particular attention should be paid to prevent undesired blockages to the transcription complex. Targeting in close proximity upstream from the TSS seems favorable, but the CRISPR complex should not be too close to create physical hindrance for the transcription complex formation, and it should also bind outside of known enhancer or transcriptional regulatory elements (TATA or CCAAT box) in the promoter. Without precise

knowledge of the regulatory elements in the sequence of the targeted promoter, empirical testing of crRNAs will remain necessary. In the extent of transcriptional activation achieved with CRISPRa, upregulation is dependent on the effect of native regulatory proteins as well as the native transcription level. When the CRISPRa system is correctly positioned, transcriptionally silent genes can be drastically upregulated, while enhanced activation of transcriptionally active genes is generally marginal.[144] Problems of incorrectly positioned CRISPR guides could be potentially solved by deploying multiple spacers to the same promoter if the chosen CRISPRa/i system supports multiplexing. To conclude, with careful design CRISPRa can be applied as a targeted transcriptional activation tool for SM discovery, bypassing the need for laborious genome editing efforts.

CRISPR-based chromatin remodeling

As discussed before, the chromatin landscape plays an important role in transcriptional regulation in filamentous fungi.[38,39,145] Since prokaryotic Cas proteins are not suited to cope with such obstacles as nucleosomes, it is expected that nucleosome-bound DNA hinders CRISPR activity. As CRISPR-based genome editing only involves a one-time event, and as the organization of chromatin is continuously changing, it is hypothesized that these spontaneous remodeling events contribute to the efficacy of CRISPR-based editing and its widespread success and applicability in eukaryotic organisms.[146] In contrast, for achieving potent CRISPRa/i transcriptional regulation at the promoter region, a persistent binding of the regulator is likely needed, which can be negatively affected by the chromatin state.

Nucleosome maps for fungal genomes are essentially undescribed. Since the chromatin organization can change depending on the cultivation conditions, it is advised to perform mapping in the same conditions as the CRISPRa/i application is planned to be executed. Fungal nucleosome maps could potentially help to identify genomic regions obscured by nucleosomes and therefore less accessible to the transcriptional complex, as well as nucleosome-free DNA regions, which are more favorable targets for CRISPR-based applications.

Nucleosome mapping has been applied for *A. nidulans* to facilitate the design of efficient protospacers for dCas9-VPR. Indeed, targeting the nucleosome-free region of a bidirectional promoter in a cryptic BGC with a single sgRNA resulted in significant transcriptional activation of genes up- and downstream of the spacer sequence.[147] Further, by targeting multiple protospacers (nucleosome-free and nucleosome-bound) synergistic activation effects were observed. For targeted chromatin remodeling, a fusion of dCas9 with the core domain of the human acetyltransferase p300 (dCas9-p300Core) has been successfully employed in mammalian cells to target enhancers regions upstream of the promoter of interest. This targeted acetylation resulted in increased expression of the downstream genes.[148] Recently, the dCas9-p300Core system has been employed in *A. niger*, where three different genes were targeted individually and successfully upregulated.[149]

Biosynthetic gene cluster refactoring

Expression of all the relevant genes of a BGC with constitutive, inducible, or synthetic promoters—thus involving major refactoring and cloning efforts—is an effective approach to characterize cryptic BGCs. Although using filamentous fungal hosts has numerous advantages (as will be discussed later), tools and expression platforms are still underdeveloped compared to other well-established species such as *S. cerevisiae*.

The promoter replacement technology is commonly used for the overexpression of a gene of interest. Selected promoters are capable of a high transcription rate under the employed cultivation conditions. Strong promoters are often selected by using transcriptome data analysis or empirical testing. Usually, these are strong constitutive promoters responsible for the transcription of housekeeping genes or other genes that are highly expressed *in vivo,* or show inducibility; e.g. *gpdA* (ANIA_08041), *glaA* (An03g06550), *pcbC* (Pc21g21380), *40S-rps8* (An0465), *tef1* (ANIA_04218).[150] Inducible promoters are found in a similar manner, but employing a well-defined chemical (alcohols, antibiotics, hormones, or carbon sources) as a potential inducer that can be added in various

amounts to repress or enhance gene expression levels.[150]

Unfortunately, individual replacement of all native promoters in a large BGC with strong promoters is an elaborate and time-consuming task, further complicated by the limited availability of well characterized fungal promoters. Nonetheless, such extensive refactoring can still be attempted with a filamentous fungal host that shows a high HR rate that facilitates recombination of DNA fragments *in vivo*.[151,152] Single promoter replacement is much more practical when it is employed to overexpress an in-cluster regulator, which in turn results in complete BGC activation with minimal engineering efforts [153,154]. Alternatively, prior to the fungal transformation the target BGC can be pre-assembled with the chosen promoters and terminators using advanced cloning methods or hosts with high HR rate, such as *S. cerevisiae*.[155] For example, the 25 kb long geodin BGC from *A. terreus* was delivered into *A. nidulans* after pre-assembly using USER fusion from 8 PCR products containing the 13 native genes, and at the same time replacing and overexpressing the transcriptional activator of the cluster.[156] Alternatively, such large genomic segments can be captured on fungal artificial chromosomes (FACs), as discussed in the following section.[157] Since fungal promoters for overexpression approaches are limited, and not every BGC contains a specific transcriptional activator to overexpress, alternative solutions are needed. Although the decreasing prices of DNA synthesis could revolutionize BGC screening by making the synthesis of entire clusters affordable, the current price levels only allow for the synthesis of smaller DNA fragments. Polycistronic expression of multiple genes has been successfully applied in filamentous fungi using only one established promoter and one terminator [158], and this could be a potential alternative for BGC refactoring. Synthetic promoters with orthogonal STF-based regulation (discussed earlier) could be used for a scaled, tunable or coordinated expression of refactored BGCs. Such systems can be delivered via genomic engineering of the native host, or by using shuttle vectors and suitable heterologous hosts.

Fungal shuttle vectors

Next to methods that require introducing genetic parts permanently into the genome of the host organism, vector-based, genome-editing–free alternatives are also available for gene expression in filamentous fungi. Fungal shuttle vectors allow the pre-assembly of genes of interest or complete BGCs in well-established model organisms like *Escherichia coli* or *S. cerevisiae*, thereby facilitating rapid cloning and subsequent delivery to the desired expression host. Since the isolation and identification of the AMA1 replicator sequence from *A. nidulans*[159], vectors bearing this sequence were shown to self-replicate in species within the genera *Aspergillus, Penicillium, Giberella*[77] as well as in *Trichoderma reseei*[160], *Lecanicillium*[161], and *Paecilomyces variotii*[162]. Telomeric sequences have also been reported to promote replication (and often integration) in various filamentous fungi like *A. nidulans*[163], *Fusarium oxysporum*[164], and *Chrysosporium lucknowense*[165]. All of these vectors can be used efficiently for rapid assembly and delivery of transcription units expressing the gene(s) of interest into the host organism. The copy number of vectors maintained within the host differs by fungal species, and it is also influenced by the strength of the selection marker or the cultivation conditions. *Aspergillus* strains were shown to maintain numerous copies of AMA1 vectors in one nucleus.[76] Since they do not integrate in the genome, these vectors are easily lost without marker selection pressure[77], which allows easy recycling of the same vector-based system.

In an impressive study, fungal artificial chromosomes based on AMA1 shuttle vectors have been used to capture the entire genome of *A. terreus* and to successfully clone all of its native BGCs in *A. nidulans,* resulting in the discovery of the astechrome biosynthetic machineries.[157] The same approach was used to clone and overexpress 56 BGCs from other *Aspergillus* species in *A. nidulans*, resulting in the discovery of 15 novel metabolites.[18] Although these shuttle vectors contained the BGCs with their native promoters, most of these compounds were not produced in the native hosts. Activation of these cryptic BGCs could be due to the presence of multiple copies of the vectors, or to the absence of native repressing factors such as epigenetic repression. Using fungal shuttle vectors

in combination with modular cloning technologies and other well characterized advanced DNA assembly tools allows rapid refactoring and validation of multi-gene expression cassettes as well as synthetic metabolic pathways.[93,96]

BGC expression using polycistronic mRNA

To allow simultaneous expression of multiple genes from one established fungal promoter, and to avoid tedious promoter replacement of all genes in a BGC, the cluster can be reconstructed using so-called "Stop-Carry On", or ribosomal "skipping", sequences between genes cloned in a sequential organization. Viral 2A peptides have been shown to promote ribosomal skipping during translation from polycistronic mRNA.[166] Since its discovery, this method has been widely applied in eukaryotes for multiple protein delivery from a single transcript. The 2A peptide sequences have been used to express the three genes of the penicillin BGC from one polycistronic mRNA in *A. nidulans.*[158] As the same promoter is driving the expression of all the genes of the BGC an equimolar production of each enzyme might be expected, which can lead to imbalances in the pathway and accumulation of toxic intermediates.[167] Some technical limitations with P2-based BGC expression are potential enzyme activity problems created by the remnants of the 2A peptide sequence at the C-termini of the proteins, validation that all genes from the transcript are effectively translated, and tedious vector construction time.[167] To solve the first issue, the additional amino acids can be removed introducing Tobacco Etch Virus (TEV) endopeptidase recognition sequences and co-expressing this peptidase in the host, and a seamless cloning step has been utilized to clone the genes of the BGC and label them with P2 and TEV recognition sequences.[167] To solve the second issue, it is possible to incorporate a split fluorescent reporter to ensure that the first and last genes are correctly translated. This advanced 2A-based expression system was applied for the heterologous expression of the austinoid BGC (~13 kb) from *A. calidoustus* and the psilocybin BGC (~7.4 kb) from *Psilocybe cubensis* in *A. nidulans.*[167]

Table 1. Transcriptional activation tools and methods for fungal biosynthetic gene clusters

Transcriptional activation method	Benefits	Drawbacks
Overexpression of BGC core gene	• Reliable transcriptional activation of the targeted gene • Limited genomic modulation needed	• Although transcription is activated, product formation is not ensured • Does not activate the complete BGC
Modulation of BGC-specific TF	• Overexpression of positive regulator can upregulate entire BGCs • Limited genomic modulation needed	• Often no cluster-specific TFs are present in a BGC • Overexpression of such TF does not guarantee transcriptional activation of the entire BGC • Other co-activators, mediators or inducers might be needed for activation
Modulation of global regulators	• Multiple BGCs are affected, resulting in higher chances for compound discovery • Limited genomic modulation needed	• Regulator needs to be identified • Global regulator targets are often unknown • Modulation can be lethal • Difficult to assign newly produced compounds to specific BGCs
Epigenome modulation	• Feeding of chemical modulators is easy to carry out • Multiple BGCs are affected, resulting in higher chances for compound discovery	• Histone modifying enzymes have to be identified and engineered • Modulation can be lethal • Difficult to assign newly produced compounds to specific BGCs
BGC refactoring	• Native regulatory system is bypassed • Episomal delivery of BGCs can lift the burden of epigenetic repression • Transcription relies solely on established promoters • Fungal SM deficient strains are available	• Requires extensive DNA cloning and/or synthesis efforts • Limited number of established promoters
Heterologous expression in non-fungal host	• Established heterologous systems and regulation tools are broadly available	• Potential problems with codon usage, available precursors, cellular trafficking, RNA splicing and post-translational modifications
STF-based BGC regulation	• Native regulatory system is eliminated or bypassed • Transcription relies on an orthogonal regulatory system • Modular features and scalable transcriptional regulation possible	• Extensive DNA cloning and/or DNA synthesis effort required • Genome editing or BGC refactoring is required • Validation (specificity, activity) of new STFs is necessary
CRISPR-based BGC regulation	• Genome editing-free transcriptional activation or repression • Rapid library construction • Various regulatory domains are available for transcriptional activation, repression or epigenetic modulation	• Extensive DNA cloning and/or DNA synthesis effort required • Genome editing or BGC refactoring is required • No established rules available for creating STF fusions • Preceding validation required (activity, specificity)

Filamentous fungi as platforms for the heterologous production of secondary metabolites

Because the great majority of fungi cannot be cultivated under laboratory conditions, model host strains are required for the heterologous expression of fungal BGCs and product identification. While common organisms as *E. coli* and *S. cerevisiae* have been successfully used for this purpose in some cases[168–173], filamentous fungi are much more suitable hosts for BGC expression for several reasons. Firstly, introns do not strictly need to be removed when cloning a putative biosynthetic gene of interest, since filamentous fungi are more likely able to process them accurately during splicing, yielding the correct mRNA. Naturally, the chances of correct splicing are higher when cloning genes from organisms that are more closely related to the host of choice.[174] The chances of a successful expression are further increased by a more ideal codon usage.[175] Secondly, fungi are more likely to produce the building blocks utilized by biosynthetic enzymes for secondary metabolite biosynthesis, because they are naturally wired for such processes. For the same reason, these hosts possess a plethora of accessory enzymes that are required for the correct functioning of the BGCs enzymes, such as phosphopantetheinyl transferases (PPTases), redox partners for P450s, prenyltransferases, and other enzymes.[10] Additionally, hosts such as *A. niger, A. oryzae* are classified as GRAS (Generally Recognized As Safe) organisms, and therefore they are suitable for the industrial production of compounds destined to be used in humans. The most commonly employed hosts for the characterization of heterologous BGCs are *A. nidulans* and *A. oryzae*[176–178], but other species have been successfully developed into platform strains, as showcased in Table 2. In the following section, we will discuss the most relevant examples and highlight their major features.

The filamentous fungus most widely used as heterologous host is by far *A. nidulans*. This species has been used for decades to study important cellular processes such as recombination, DNA repair, and chromatin regulation[179–181], because it can be easily manipulated and cultivated in the lab. This has led to the development of several platform strains that have been engineered to characterize

and overexpress heterologous genes and, ultimately, produce natural products. The most interesting platform strains, showcased in Table 2, are derived from a triple auxotrophic strain called A1145.[182] This strain also carries a deletion of the *nkuA* gene (homolog of human *ku70*) that renders NHEJ DNA repair less favorable, facilitating precise genomic integration of heterologous genes via HR. A1145 has been successfully used to elucidate a diverse range of biosynthetic pathways belonging to all major classes of SMs.[183–186] Despite these attractive features, *A. nidulans* also has a major downside. It is a prolific producer of SMs[176,177] that typically yields a crowded chromatographic background, which can render the identification of new metabolites a cumbersome task. To partially overcome this problem, strains with a cleaner SM background have been developed. In one instance, the BGCs responsible for the biosynthesis of sterigmatocystin and emericellamids, two major classes of compounds produced by *A. nidulans*, were deleted. This allowed the identification of an important intermediate of the cholesterol-lowering compound zaragozic acid A in an engineered strain, previously overshadowed by the host metabolites.[187]

A similar strain—LO4389—where only the sterigmatocystin pathway was deleted, was used for the identification of 6 polyketides from *A. terreus* along with the complete reconstitution of the *A. terreus* asperfuranone pathway[188], underlining once again the potential of such cleaner platform strains. Following the success of such trials, the same authors reported the construction of strain LO8030 where eight of the most highly expressed BGCs were deleted, resulting in a considerable reduction of the genome size.[189] Despite that, the strain showed no significant defects in growth. Additionally, not only did the strain offer a minimal background, but it also benefitted from a higher availability of SM precursors, as demonstrated by the synthesis and detection of the previously undiscovered metabolite aspercryptin.[189] Recently, LO4389 was used to construct two new strains that possess genetic features that are especially advantageous for the expression of entire biosynthetic pathways. In these strains, up to 6 or 7 genes of interest (GOIs) can be placed under the control of the native regulatory elements of the asperfuranone pathway, whose genes have been removed, while

the inducible promoter P*alcA* controls the major TF regulating the BGC pathway. This elegant approach allows for the induction of entire heterologous BGCs upon the addition of methyl ethyl ketone, and its potential was demonstrated by the successful production of citreoviridin, mutilin, and pleuromutilin.[152]

Another common choice as a cell factory for the identification and production of SMs is *A. oryzae*. This fungus plays an important role in food manufacturing in Asia, where it is widely used for the production of alcoholic beverages and fermented products such as soy sauce and miso. *A. oryzae* contains numerous BGCs in its genome, many of which are also found in the toxins-producing species *A. flavus*, which is a prolific producer of natural products. In fact, the species are so closely related that it is believed that *A. oryzae* is actually a product of the domestication of A*. flavus.*[190] However, *A. oryzae* produces only a few endogenous SMs, which makes it a perfect host for heterologous production.[177] The most common platform strain is the quadruple auxotroph NSAR1[191] which offers great versatility and does not require the need of expensive additives for the selection of the transformants. NSAR1 has been used successfully by many researchers to elucidate diverse biosynthetic pathways[192–195]. Despite the fact that its use is relatively more recent than *A. nidulans,* an extensive toolkit is now available to transform *A. oryzae* and express heterologous genes[196,197]. Of particular interest is a system of vectors—pTYargB/niaD/adeA/sC-eGFPac—that carry an inducible expression cassette (under control of the *amyB* promoter and terminator) and three constitutive cassettes. There are four versions of these vectors, each carrying a different selection marker, that can be co-transformed to allow the overexpression of up to 16 heterologous genes in the recipient NSAR1 strain[197]. Although *A. oryzae* already offers a clean SM background, it produces a relatively abundant compound called kojic acid. The presence of kojic acid can complicate the purification of metabolites of interest and interfere with structural characterization. Deletion of the gene *kojA*, that encodes a crucial oxidoreductase for the synthesis of kojic acid, resulted in an astoundingly clean SM background and easy detection of the metabolite of interest[198]. Another platform strain that merits attention is the triple auxotroph

NSlPD1[199]. The advantage of this particular strain over NSAR1 and its derivative strains is the deletion of the *ligD* gene, which facilitates HR-mediated genetic engineering (analogously to the deletion of *nkuA* in *A. nidulans*). NSlPD1 was successfully engineered to generate a strain that produces higher titers of kojic acid and uses cellulose as starting material[200].

Two other well-known Aspergilli that have been explored as hosts for the biosynthesis of natural products are *A. niger* and *A. terreus*. The former has been used for the efficient production of the depsipeptides enniatin[201], beauvericin, and bassianolide[120]. These compounds show high insecticidal activity[202] and some have been proposed as potential candidates for the treatment of HIV infections.[203] *A. terreus* is less commonly used as a heterologous host, but its tremendous natural capabilities as a producer of SMs[204] suggest that it could represent a worthy alternative to other fungal species, especially for the production of polyketides. In fact, this species is widely used for the industrial production of lovastatin[205], an essential pharmaceutical that is used to treat high blood cholesterol. In recent years, platform strains of *A. terreus* have been used to elucidate the biosynthetic pathway of the mycotoxin flavipucine[206], and to generate a high-performance strain capable of producing high titers of monacolin J, a key precursor to the synthesis of semi-synthetic statins.[207]

Another important fungal workhorse for industrial applications is *P. rubens,* which is used for the production of penicillin, cephalosporin, and other β-lactam antibiotics. To increase the titers of penicillin produced, early strains of *P. rubens* have been subjected to decades of random mutagenesis and selection processes, collectively known as the classical strain improvement program. This has also led to increased capabilities to grow in submerged cultivation conditions and in defined media, features that are very desirable in the industry.[208–210] The CSI program also led to a major reduction of the expression and/or mutational inactivation of non-penicillin BGCs. One of the industrial penicillin producer strains was recently engineered to abolish production of β-lactam antibiotics, with the assumption that this strain would retain its metabolic capabilities while offering a cleaner background. Indeed, this particular strain was used to heterologously

express the compactin BGC from *P. citrinum*, together with an engineered cytochrome P450 from *Amycolatopsis orientalis*. The newly engineered strain was able to catalyze the final hydroxylation step of compactin to the cholesterol-lowering drug pravastatin, with an impressive yield of more than 6 g/L in pilot scale fermentations.[211] This idea was further explored even more recently, when the same β-lactam-deficient strain was used as a template to generate a quadruple deletion strain in which alongside the penicillin BGC three major BGCs (chrysogine, fungisporin, and roquefortine) were removed, resulting in a considerably clean SM background, ideal for natural product production.[151] As proof of concept, the SM-deficient platform strain was used to reintroduce the penicillin BGC, resulting in restored production of the antibiotic, and to overexpress an endogenous PKS (PKS17), leading to the production of YWA1, a common precursor to fungal pigments. Additionally, the strain was used for the successful reconstitution of the calbistrin BGC from *Penicillium decumbens*, which resulted in the heterologous production of decumbenone A, B, and C [151]. This strain is also devoid of the *hdfA* gene (homolog of human *ku70*) and is therefore suitable for HR-mediated genetic engineering. These results highlight the *P. rubens* 4xKO strain as a valuable option for SM research.

Another filamentous fungus worth mentioning is *T. reesei*. For decades, this organism has been used in industry for its astonishing ability to produce cellulolytic enzymes such as cellulases and hemicellulases[212], but it never attracted natural product researchers, probably due to the broad availability of other hosts and a lack of well-developed synthetic biology tools. Recently, a strain which carries deletions for the genes *tmus53* (*ligD* homolog) and *pyrG* was engineered.[213] Analogously to other fungal hosts, the Δtmus53 deletion facilitates HR-mediated targeted gene integration, while ΔpyrG allows easy selection through complementation of uracil/uridine auxotrophy. These features raise interest for *T. reesei* as a potential natural product producing host. In fact, this particular strain has already been utilized to investigate the endogenous biosynthetic pathway of sorbicillinoids.[214] What could set this platform apart and make it a concrete option for industrial applications is its natural ability to

degrade and thrive on cellulosic material, which could lead to the production of natural products starting from biomass material, such as agricultural waste.

Undoubtedly, the fungal platforms discussed above provide a rich choice for researchers who want to investigate unknown biosynthetic pathways or produce industrially relevant metabolites. Nevertheless, despite the broad availability of hosts, not all species might be capable to produce the desired natural product, and even when they are, it is very likely that one species performs better than another in terms of yield. This is difficult to predict and engineering more species at once to optimize production can be extremely time-consuming as well as costly. To reduce the workload and facilitate simultaneous cloning and screening of more hosts, a group of researchers recently developed the first multispecies fungal platform for heterologous gene expression.[215] The first version of this system, called DIVERSIFY, is based upon four *Aspergillus* species: *A. nidulans*, *A. oryzae*, *A. niger*, and *A. aculeatus.* Each individual species was first engineered to contain in the genome a "common synthetic gene integration site" (COSI), which encodes a reporter gene for white/blue selection placed under the control of a constitutive promoter. Additionally, the COSI contains two 500 bp sequences flanking the reporter cassette that can be used for HR-mediated target gene replacement, whereby GOIs can be easily inserted and overexpressed. Since the COSI is equal in each recipient strain, only one integration cassette has to be designed and built. As a proof of concept, the DIVERSIFY platform has been successfully used to overexpress a fluorescent reporter (mRFP) and cellobiohydrolase, and for the production of 6-MSA, a model polyketide.[215] Because many of the synthetic biology tools developed for fungi are readily adaptable to other species, this platform can be expanded with other hosts in the future.

Table 2. Examples of fungal expression platforms for the production of natural products and the characterization of biosynthetic gene clusters.

Species	Platform strains	Genotype	Examples of NP	References
Aspergillus nidulans	A1145	*pyrG89*; *pyroA4*; *nkuA*::*argB*; *riboB2*	Flavunoidine	186
			Myceliothermophin	183
	A1145 ΔSTΔEM	A1145 Δ*stc*-BGC, Δ*eas*-BGC	Zaragozic acid A precursor	187
			Trihazone A-F	216
	LO4389	A1145 Δ*stcA-stcW*	Asperfuranone	188
	LO8030	A1145 Δ*stc*-BGC, Δ*eas*-BGC, Δ*afo*-BGC, Δ*mdp*-BGC, Δ*tdi*-BGC, Δ*aus*-BGC, Δ*ors*-BGC, Δ*apt*-BGC	Aspercryptin	189
			Felinone A	37
	YM87 & YM137	LO4389 AN1029::P*alcA*-AN1029; AN1036-AN1032(31)::*AfriboB*	Citreoviridin	152
			Mutilin	152
			Pleuromutilin	152
Aspergillus oryzae	NSAR1	*niaD*$^-$, *sC*$^-$, Δ*argB*, *adeA*$^-$	Strobilurin	194
			Paxilline	192
			Erinacine Q	193
	NSARΔK	NSAR1 Δ*kojA*	Pretenellin A	198
	NSPlD1	*niaD*$^-$, *sC*$^-$, *pyrG*$^-$, *ligD*$^-$	Kojic acid	200
Aspergillus niger	AB1.13	*pyrG1*, *prtT*$^-$	Enniatin	201
			Beauvaricin	120
			Bassianolide	120
Aspergillus terreus	Δ*akuB*	SBUG844 Δ*akuB*::*hphR*	Isoflavipucine	206
			Dihydroisoflavi-pucine	206
	HZ03	Δ*ku80*::*ptrA*, Δ*pyrG*	Monacolin J	207
Penicillium rubens	4xKO	Δ*hdfA*, Δ*pen*-BGC, Δ*chy*-BGC, Δ*roq*-BGC::*amdS*, Δ*hcpA*::*ble*	Penicillin	151
			Decumbenone A-C	151
Trichoderma reseei	Δ*pyrG*	QM6a Δ*tmus53*, Δ*pyrG*	Sorbicillinoids	214

Clearly, filamentous fungi are powerful instruments for the elucidation of biosynthetic pathways and the production of SMs. In many cases, though, the desired compounds are produced at very low yields which are unsuitable for commercial applications. The development of a great array of hosts, each with specific benefits, as well as multispecies platforms that allow fast and simultaneous screening of several fungi with reduced workloads, will offer

researchers the tools to readily optimize the production yield of metabolites of interest. Ultimately, this is necessary to engineer efficient fungal cell factories that are ready to be employed at an industrial scale.

Concluding remarks

Thanks to the rapidly expanding number of filamentous fungal genome sequences and the advanced bioinformatics tools that are now available, it has become obvious that filamentous fungi represent an untapped reservoir of natural products. Each genome contains a high number of BGCs for which the product has not been identified, and many of these BGCs are transcriptionally silent under laboratory conditions. The products of these clusters can be unearthed with the combined efforts of bioinformatics, chemistry, and synthetic biology, to reveal new chemistries and biological activities of interest. A major challenge, however, remains prioritizing these BGCs for their potential value since the bioinformatics tools available at the moment cannot reliably predict the resulting products. Hence, much relies on laborious empirical testing, whereby many BGCs have to be expressed to screen for bioactive compounds. Here, we have discussed conventional tools and the development of new synthetic biology tools that aid in the transcriptional activation of silent BGCs in filamentous fungi, therefore offering new approaches for compound discovery (Table 1 and Figure 2).

Modulating global regulatory systems does not require prior knowledge regarding the cluster-specific regulation mechanism of a given BGCs, and although numerous genes can be affected when using this approach, transcriptional activation of BGCs of interest and subsequent production of new metabolites are not guaranteed. Instead, overriding the native regulatory system of the cluster can result in a more direct transcriptional activation. Such approaches involve partial or complete BGC refactoring, and require a deep level of understanding about the number of genes in the BGC, and/or its cluster specific regulators. Refactoring approaches can take place within a host which supports the assembly of numerous DNA fragments, often achieved via homologous recombination or advanced synthetic cloning methods. The only approach that ensures

transcriptional activation of an entire BGC is promoter replacement of all its genes, but this is generally a laborious and cumbersome task. Furthermore, the number of established strong fungal promoters and fungal selection markers is still limited, and this represents a bottleneck towards rapid or consecutive genomic modifications. An alternative to serial replacement of promoters could be the utilization of promoters from BGCs which are transcriptionally active in the selected host, or synthetic promoters containing regulatory elements for orthogonal regulatory systems such as STFs. The more recent CRISPRa systems further increase the number of available activator tools adding a new layer of control, since such systems do not require BGC refactoring anymore.

Since genetic manipulation and precise engineering of a non-model or wild type fungal strains is challenging—mainly due to the low rate of HR and difficulties to grow these organisms in laboratory conditions—the most versatile method is to express BGCs in an established heterologous host. Ideal host strains are those in which it is easy to perform genetic manipulation, that are convenient to cultivate at different scales, for which compatible genetic tools are available, and convenient downstream processing steps have been established such as rapid compound screening and a clean metabolite spectrum. A combination of advanced transcriptional activation tools and established expression hosts can ensure reliable, targeted transcriptional activation and efficient methods for compound identification.

Although new host strains and tools are continuously being developed for the characterization of cryptic BGCs in filamentous fungi, high-throughput BGC screening remains a major challenge. Automatized engineering of protoplasted filamentous fungi using microtiter plates and robotic liquid handling robots have been successfully established[217], as well as fully-automated microscale bioreactor cultivations[218], but working with BGC-coding DNA is demanding. The large size, the numerous genes, and the costs for total cluster DNA synthesis are limiting factors for rapid assembly and screening of numerous BGCs. In the future, the combination of emerging genetic tools, tailored heterologous hosts with high metabolic capacity, and automated systems, will facilitate the

development of highly efficient, targeted, multiplexing-compatible transcriptional activation applications for novel natural product discovery. This, coupled with the development of bioinformatics tools that are able to prioritize the most valuable BGCs within genomic sequences, will revolutionize the field and eliminate time-consuming and costly wet lab procedures, while starkly increasing the chances of identifying novel and potent bioactive compounds.

Abbrevations

AD, activation domain; CP, core promoter; DBD, DNA-binding domain; STF, synthetic transcription factor; UAS, upstream activating sequence

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

LM and RI wrote the manuscript with the critical feedback of AJMD and RALB. LM and AJMD conceived the manuscript. AD supervised the manuscript. RALB co-supervised the manuscript.

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CHAPTER 2

Synthetic control devices for gene regulation in *Penicillium chrysogenum*

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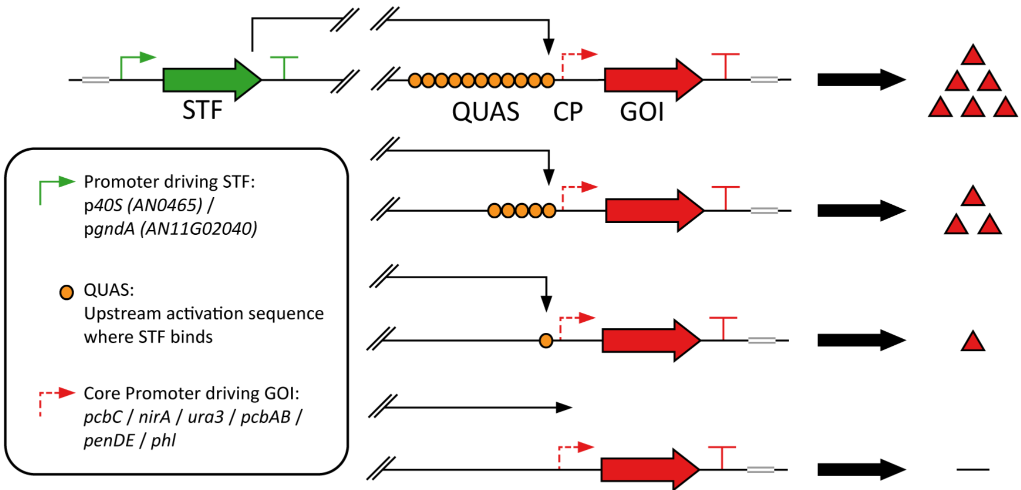
Graphical abstract



Abstract

Background

Orthogonal, synthetic control devices were developed for *Penicillium chrysogenum*, a model filamentous fungus and industrially relevant cell factory. In the synthetic transcription factor, the QF DNA-binding domain of the transcription factor of the quinic acid gene cluster of *Neurospora crassa* is fused to the VP16 activation domain. This synthetic transcription factor controls the expression of genes under a synthetic promoter containing quinic acid upstream activating sequence (QUAS) elements, where it binds. A gene cluster may demand an expression tuned individually for each gene, which is a great advantage provided by this system.

Results

The control devices were characterized with respect to three of their main components: expression of the synthetic transcription factors, upstream activating sequences, and the affinity of the DNA binding domain of the transcription factor to the upstream activating domain. This resulted in synthetic expression devices, with an expression ranging from hardly detectable to a level similar to that of highest expressed native genes. The versatility of the control device was demonstrated by fluorescent reporters and its application was confirmed by synthetically controlling the production of penicillin.

Conclusions

The characterization of the control devices in microbioreactors, proved to give excellent indications for how the devices function in production strains and conditions. We anticipate that these well-characterized and robustly performing control devices can be widely applied for the production of secondary metabolites and other compounds in filamentous fungi.

Keywords: synthetic expression system, gene regulation, *Penicillium chrysogenum*, synthetic gene cluster, secondary metabolite production, hybrid transcription factor

Background

Synthetic biology has revolutionized metabolic engineering and takes the exploitation of industrial microorganism to a new level by enabling fine-tuning of gene expression and control of entire pathways. Recent advances such as CRISPR/Cas9 technologies accelerate strain construction and enable complex pathway engineering of also more challenging hosts.[1] The metabolic diversity and the wide range of ecological niches that fungi inhabit gives them a great potential as sources of novel enzymes, and the use of fungi in white and red biotechnology is well established.[2] Thus, there is a great demand for synthetic biology tools for fungal cell factories.

Filamentous fungi such as *Penicillium chrysogenum* produce a variety of interesting secondary metabolites (SMs), compounds that are not essential for growth, but typically possess bioactivities that are of great value to medicine, agriculture and manufacturing. *P. chrysogenum* is well known to produce β-lactam antibiotics, but it naturally produces a wide variety of SMs, and it has also been engineered to produce heterologous compounds.[3] Still, many SM clusters are not expressed under laboratory conditions and may need to be activated or heterologously expressed before the SMs can be obtained.[4] A further challenge is that many SMs genes are carbon catabolite repressed.[5] The number of sequenced filamentous fungi has lately seen a great increase, highlighting the need for orthogonal tools to explore the enormous potential of new SM biosynthetic clusters and their respective natural products. So far, no orthogonal expression systems have been used for activation of entire gene clusters in fungi and the challenge in expression of silent SM clusters forms a bottleneck in exploring the diversity of natural SM products. Therefore, the development of efficient expression devices is of great importance.

A number of promoters have been characterized for *P. chrysogenum*[6] and a couple of expression systems for protein production and secretion with *Penicillium* have been reported.[7,8] The use of a promoter and its transcription factor from a native SM cluster for the production of high amounts of heterologous SMs, has been demonstrated in *Fusarium heterosporum*.[9] Similarly, a heterologous fungal

expression system based on regulatory elements of the terrein gene cluster of *Aspergillus terreus* was demonstrated in *A. niger*.[10] Synthetic gene expression systems consisting of heterologous and hybrid transcription factors (TFs) composed of different DNA-binding and activation domains have previously been demonstrated in Aspergilli[11–14], *Ustilago maydis*[15] and *Trichoderma reesei*[13]. The systems developed were induced by doxycycline/tetracycline[11,12,15,16] or estradiol[17] and while widely useful for proof-of-concept studies, the need of an inducer provides a physiological complication[18] and potential commercial hindrance.[19] Recently synthetic expression systems developed by Rantasalo *et al*.[13], where hybrid transcription factors were expressed using a transcriptionally active core promoter (CP) (providing a low constitutive expression) instead of a full-length promoter. These systems were shown to be functional in several yeasts and two filamentous fungi.

An increasing number of promoter libraries have been designed for prokaryotes and yeast, by random sequence modifications or by rational approaches, including introduction of various upstream activating sequence (UAS) elements[20] or evaluating different CPs.[19,21] TFs conferring specific activation/repression mechanisms interact with designated UAS elements, but a CP (sometimes called minimal promoter) sequence is required to recruit general transcription factors and the RNA polymerase II for transcription initiation (reviewed by Juven-Gershon and Kadonaga[22]). The CP is the minimal portion of the promoter required to initiate transcription, typically containing the site for start of transcription, the polymerase binding site and general transcription factor binding sites, such as the TATA and CCAAT boxes and the initiator element[22]. These CP elements are found in some but not all promoters and the sequence – function relationship of these elements remains unclear. CPs of the *nirA*[17] and *gaaC*[13] genes of *A. niger* and of the *gpdA*[12] gene of *A. nidulans*, as well as the *ura3* gene of *Saccharomyces cerevisiae*[17] have been demonstrated to function in Aspergilli. The transcription start sites have been described for the penicillin biosynthesis gene cluster of *P. chrysogenum*[23] but so far there are no CPs demonstrated in this fungus.

The Q-system is a binary system for transgene expression, originally developed for *Drosophila* and mammalian cells[24,25], that has also been demonstrated in *Caenorhabditis elegans*[26], zebrafish[27] and malaria mosquitos[28]. The Q-system utilizes regulatory genes from the *Neurospora crassa* quinic acid gene cluster. The *N. crassa* quinic acid genes contain binding sites named QARE (QA response element)[29], referred to as QUAS when used in synthetic expression systems. Here, a synthetic expression system was developed for *P. chrysogenum*, by exploring components from the Q-system.[30] In this system, the synthetic TF (STF), consisting of the QF (qa-1F) DNA-binding domain (DBD) from the TF that regulates the quinic acid gene cluster of *N. crassa* which was fused to the *Herpes simplex* virus VP16 activation domain[31] and GFP with the SV40 nuclear localization signal (NLS)[32]. We demonstrated the function of this system by fluorescent reporters and showed that the production of penicillin could be controlled by introducing the QUAS sequences and the STF in the penicillin biosynthesis gene cluster. Taken together, our control device can serve as an excellent tool for studying and increasing fungal SM production and expression of other genes of interest.

Results and discussion

Engineering of production hosts requires robust and predictably performing gene expression tools. In this study, we set out to establish such tools for *P. chrysogenum* and to demonstrate their utility for the production of penicillin, implementing synthetic regulation for a SM cluster.

Design of synthetic control devices

In order to design synthetic control devices for defined strength and expression profiles, components of the Q-system[30] were adapted for *P. chrysogenum*. The Q-system was chosen, as the DNA sequence to where the QF TF binds was relatively long (16 bp), which is needed in order to minimize pleiotropic effects and ensure a tight control. The control devices are defined as genetic systems where a STF controls the expression of a gene under a synthetic

promoter containing a core promoter (CP) and binding sites for the synthetic transcription factor (STF). The strength of the control device is determined by: 1) the strength of expression of the STF; 2) the UAS element, which is the TF-specific binding site placed upstream of the CP; 3) the affinity of the DNA binding domain (DBD) of the TF to its UAS sequence; 4) the capacity of the activation domain (AD) to recruit the transcription machinery; and 5) the CP, which is necessary for assembly of the general transcription machinery and for initiation of transcription. In this work, the first three elements were investigated (Figure 1).

The control devices consist of two transcriptional units, the donor for expression of the STF and the recipient with a reporter demonstrating the activity of the synthetic promoter (Figure 1). This construct was integrated at the genomic site where the penicillin cluster normally is located in *P. chrysogenum*. In the strain used, DS68530, all the penicillin clusters had been removed.[33] In the control devices, the STF contained the DBD of the QF TF, fused to the VP16 AD and a GFP with an NLS (Figure 1). Control devices with a modified QF AD fusion with QF DBD (QF2[25]), appeared to possibly be toxic for *Penicillium*, as no correct transformants were obtained despite numerous trials. The GFP of the STF serves as an internal control which allows for corrections in growth and

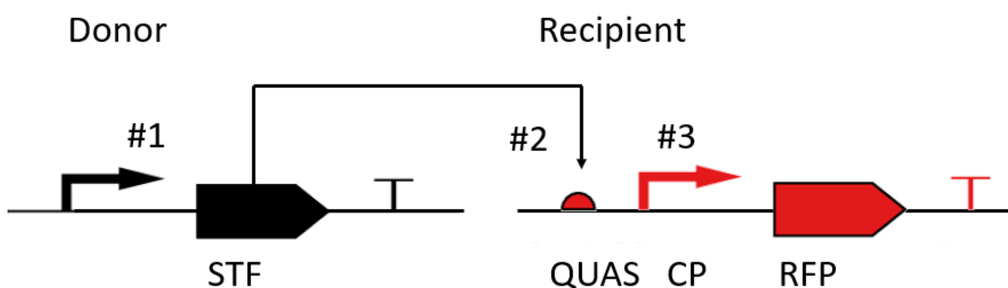


Figure 1. Schematic presentation of the control devices consisting of the donor (in black) and recipient (in red). The STF contained the QF DBD and the VP16 AD tagged with GFP-NLS, transcribed under the P40S or PgndA promoter (#1, black arrow). Control devices with 1, 5 and 11 QUAS elements (#2, red half circle) preceding various CPs (#3, red arrow) were evaluated. T's present terminators of the expression cassettes. Elements varied are indicated by numbers and explained in Table 1.

biomass differences. The STF controls the expression of the RFP reporter under a synthetic promoter containing QUAS elements upstream of a CP. CP strains (strains 4,5,7-11) express the donor with the STF but lack the QUAS elements upstream of the CP in the receiver. Six different CPs and three different QUAS elements were tested. In addition, two different promoters, P*40s* and P*gndA*, were used for expression of the STF. Strains characterized by measurement of fluorescence of control devices are listed in Table 1.

Table 1. Strains characterized by fluorescence measurements. Elements varied are marked with numbers in Figure 1. The STF transcribed under the P*40S* (An11g02040) promoter (#1) or the P*gndA* (AN0465) promoter (strains 5,18-20). All strains were derived from DS68530.

No.	Description of strain	Number of QUAS elements (#2)	Promoter for expression of RFP (#3)	Gene associated with promoter for expression of RFP
1	40s_1xQ_pcbC	1	*Pc*_pcbC CP	Pc21g21380
2	40s_5xQ_pcbC	5	*Pc*_pcbC CP	Pc21g21380
3	40s_11xQ_pcbC	11	*Pc*_pcbC CP	Pc21g21380
4	40s_pcbC_CP	-	*Pc*_pcbC CP	Pc21g21380
5	gndA_pcbC_CP	-	*Pc*_pcbC CP	Pc21g21380
6	40s_5xQUAS	5	-	-
7	40s_pcbAB_CP	-	*Pc*_pcbAB CP	Pc21g21390
8	40s_penDE_CP	-	*Pc*_penDE CP	Pc21g21370
9	40s_phl_CP	-	*Pc*_phl CP	Pc22g14900
10	40s_nirA_CP	-	*An*_nirA CP	AN0098
11	40s_ura3_CP	-	*Sc*_ura3 CP	YEL021W
12	40s_5xQ_pcbAB	5	*Pc*_pcbAB CP	Pc21g21390
13	40s_5xQ_penDE	5	*Pc*_penDE CP	Pc21g21370
14	40s_5xQ_phl	5	*Pc*_phl CP	Pc22g14900
15	40s_5xQ_nirA	5	*An*_nirA CP	AN0098
16	40s_5xQ_ura3	5	*Sc*_ura3 CP	YEL021W
17	40s_5xQ_reverse_pcbC	5	*Pc*_pcbC CP	Pc21g21380
18	gndA_5xQ_pcbC	5	*Pc*_pcbC CP	Pc21g21380
19	gndA_5xQ_nirA	5	*An*_nirA CP	AN0098
20	gndA_5xQ_ura3	5	*Sc*_ura3 CP	YEL021W
21	40s_pcbC full	-	*Pc*_pPcbC	Pc21g21380
22	40s_pcbAB full	-	*Pc*_pPcbAB	Pc21g21390

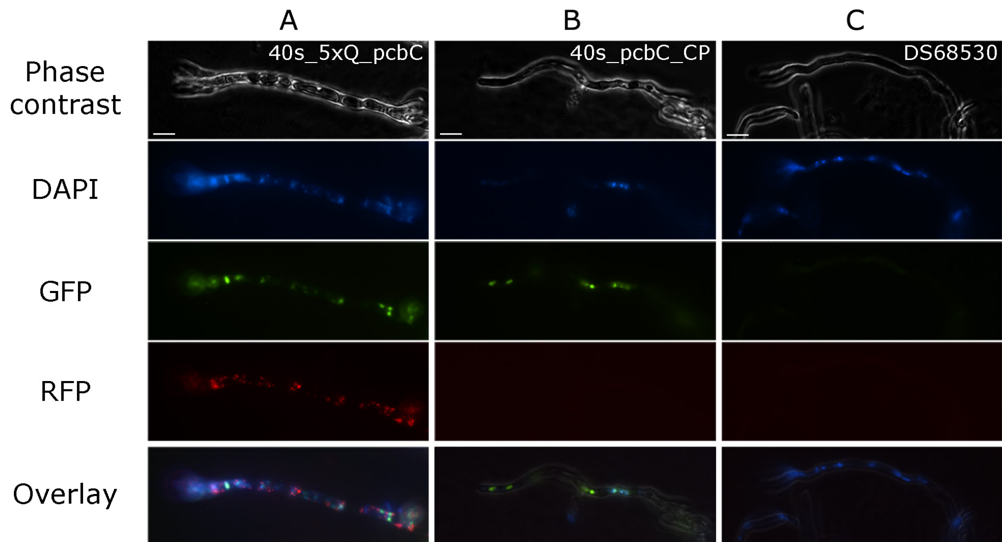


Figure 2. Fluorescence microscopy images of a filament of strain no.2; 40s_5xQ_pcbC (panel A) expressing the STF and RFP under a synthetic promoter containing 5xQUAS upstream *Pc*_ pcbc CP, strain no.4; 40s_pcbC_CP (panel B) expressing the STF but no RFP as there are no QUAS binding sites upstream of the *Pc*_pcbC CP, and the parent strain DS68530 (wt, panel C) not expressing fluorescent proteins. Scale bars represent 10 μm.

The control devices can be easily visualized due to the fluorescent protein reporters with different localization tags. The STF containing GFP with an NLS tag was localized in the nucleus and the RFP with the SKL tag[34] localizes to peroxisomes of the cells. Upon fluorescence microscopy imaging of strains expressing the control devices but lacking the QUAS elements upstream of the CP, only GFP was seen (Figure 2, panel B), whereas strains with QUAS elements had green fluorescent nuclei and red fluorescent peroxisomes (Figure 2, panel A). The nuclear localization of GFP was confrmed by DAPI staining (Figure 2, panel C). The fluorescent imaging confirmed that all control device encoding genes were expressed and that the control device worked as designed.

The BioLector microbioreactor system with online monitoring of scattered light and fluorescence was used for assessing the performance of the control devices in *P. chrysogenum*. This system has previously been used to characterize bacterial[35] and yeast[36] fermentations as well as expression of fluorescent proteins

under control of various promoters in *P. chrysogenum*[6]. Initially, we validated that no clear difference in exponential growth rate was seen among the strains evaluated and the host strain (Figure 3a and Supp. Figure S1). The exponential growth rate determined during the first 60 h of cultivation was 0,031 ± 0,002 for all strains. An increase in biomass was observed during the first ~80 h of the cultivation, after which the biomass remained constant or even decreased. None of the strains characterized in this study demonstrated any visible physiological changes during growth on liquid or solid medium. At the end of some of the cultivations, the mycelia clearly formed clumps, which likely explains the variability between some cultures seen after 80 h. It should be noted that the correlation between optical density and biomass concentration of filamentous fungi is linear only during the exponential growth phase (reviewed by Gibbs *et al*.[37]). Morphological changes after substrate depletion were for *Kluyveromyces lactis* cultures reported to influence biomass measurement[38], thus this is likely to also affect the late measurement of biomass for *P. chrysogenum* cultures.

The consumption of carbon sources of the growth medium (containing 5 g/l glucose and 36 g/l lactose) was measured after 24 and 120 h of cultivation for a few strains. After 24 h, all glucose was consumed, but around 12 g/l of lactose was still left in the medium after 120 h. The control device strains expressing fuorescent proteins were also tested in medium with glycerol or glycerol and lactose as carbon source. Glycerol is a non-fermentable carbon source that does not lead to glucose repression, while lactose is commonly used for production of SMs in fungi.[3,5,6] While growth on glycerol was challenged and no difference in expression of the control devices tested was seen in medium with glycerol and lactose, we did not pursue the testing of the production strains in diferent media. The growth of all the strains in the Biolector platform was reproducible and the biomass formation was not influenced by expression of any of the control devices tested.

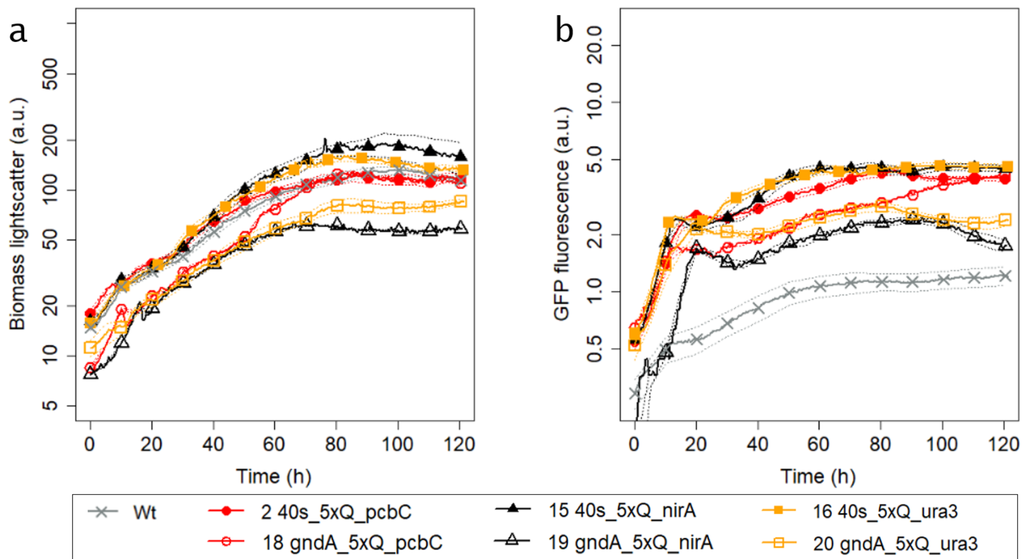


Figure 3. Development of biomass (a), GFP fluorescence (b), GFP fluorescence/biomass over time of selected *P. chrysogenum* strains containing different synthetic control devices, shown on logarithmic scales. Strain numbers and core promoters of strains are marked in the figure legend. The promoter used for expression of the STF was P*40S* or P*gndA* (marked in legend). Background fluorescence of parental strain (Wt) shown in grey (x plot symbols). Solid lines indicate mean values for at least 3 independent cultures, the dashed lines show the standard error. For data of all strains, see Supp. Figure S1.

Tuning expression by varying the expression of the STF

Two different promoters were used to drive expression of the STF, the promoter of An11g02040 (P*gndA*) and of AN0465 (P*40*S, Figure 3b, Table 1). Both promoters originate from *A. nidulans* and were previously validated in *P. chrysogenum.*[6,39] The constructs with P*40S* for expression of the STF (strains 2, 15 and 16) gave 2-3 times higher expression of RFP/GFP, compared to the construct where P*gndA* was used (Figure 4, strains 18-20) and were therefore chosen for further work. During the time interval of 40-80 h, the expression of GFP under P*40S* was approximately 1.5-2x higher than the expression under P*gndA*. This showed that the control device functions as an expression amplifier, in line with earlier observations in *S. cerevisiae*.[19]

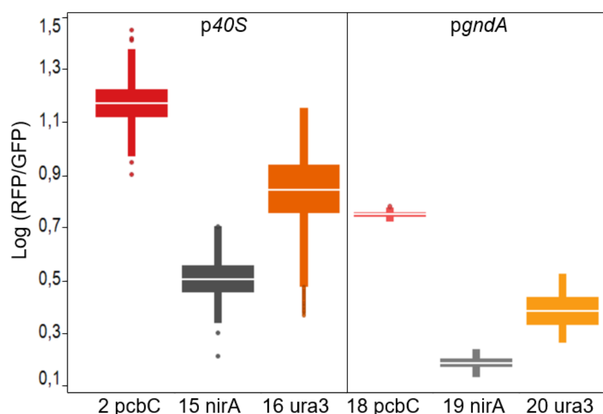


Figure 4. Ranking of the expression of 6 control devices. The activity is expressed as the logarithmic values of the averaged RFP/GFP fluorescence ratios measured during the time window of 40-80 h of growth in the BioLector system. All devices contain 5xQUAS; promoters for expression of the STF (on top) and CPs (on bottom) marked in figure. Box plot shows data of at least 3 independent cultures.

The strength of the synthetic promoters (as measured by RFP fluorescence) was determined relative to the GFP expression of the strains to avoid variance caused by differences in growth. The expression of GFP was similar among the various strains with the same promoter for expression of the STF (Figure 3b). The expression of GFP per biomass increased during the initial growth phase that lasted 10 – 20 h, depending on the strain and initial biomass concentration (Figure 5a), after which the relative GFP expression decreased as more biomass was formed. The expression of GFP under P*gndA* was somewhat lower and showed a greater variability compared to the expression under P*40S* that was very similar among the different strains (Figure 3b).

For the control device to function as anticipated, CPs should not be active by themselves, but the expression of RFP should be solely dependent on the expression of the STF. Furthermore, the QUAS element should not induce any expression by itself. The criteria set for the control devices were met: a strain with 5xQUAS elements upstream of the reporter showed no expression of RFP (Figure 5b, grey stars) and the strains lacking QUAS elements showed no or extremely weak (strain with the *An*_nirA CP) expression of RFP (figure 5b, grey triangles with an x).

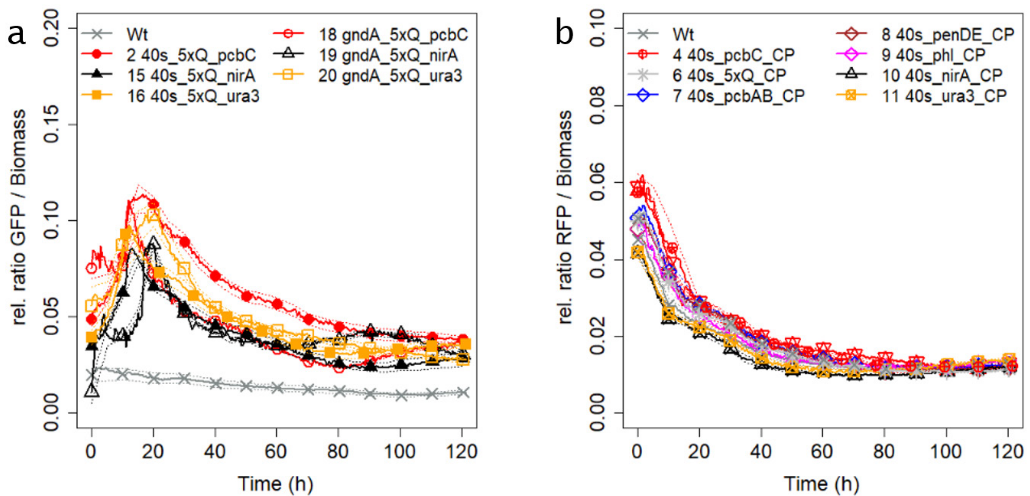


Figure 5. Relative ratio of GFP (a) or RFP (b) per biomass over time of selected *P. chrysogenum* strains containing different synthetic control devices. Strain numbers and core promoters of strains are marked in figure legend. The promoter used for expression of the STF was P*40S* or P*gndA* (marked in legend). Background fluorescence of parental strain (Wt) shown in grey (x plot symbols). Solid lines indicate mean values for at least 3 independent cultures, the dashed lines show the standard error. For data of all strains, see Supp. Figure S1.

Tuning the strength of expression of the STF by varying the CP or number of QUAS elements

No CPs have previously been identified or validated in *P. chrysogenum*. Here, the 200 bp upstream region of the ATG of the penicillin cluster genes (Pc21g21370; *penDE*, Pc21g21380; *pcbC*, and Pc21g21390; *pcbAB*) or Pc22g14900 (*phl)* was assessed as putative native CPs. *Phl* encodes a phenylacetyl-CoA ligase, involved in penicillin G and V production.[40] The CP sequences contain many putative CP elements (see Supp. Table S1) but no apparent similarities and they do not align. Nucleosome occupancy heatmaps of the receiver parts of the control devices drawn according to Kaplan *et al*.[41] were not found to correlate with the activity of the CPs (see Supp. Figure S2).

All CPs tested were shown to be functional as CPs in *P. chrysogenum* (Figure 5a). By themselves (in strains 4, 5, 7-11, Figure 5b) the CPs did not drive expression of RFP, but together with QUAS elements placed upstream, they

formed functional synthetic promoters. In promoters with the QUAS element upstream the CPs, the constructs containing the CPs of *pcbC* and *phl* gave the highest expression, whereas the constructs with the CPs of the other penicillin cluster genes, *pcbAB* and *penDE* gave a maximal expression that was around 10x lower than the expression of the construct with the pcbC CP (Figure 6a).

The expression of RFP under the control of 5xQUAS elements upstream of the *Sc*_ura3 CP was around twice as high as in a strain with the *An*_nirA CP downstream of the 5xQUAS element (Figure 6a). This is in line with observations for *A. niger* strains, where these CPs were used in estradiol responsive constructs; the *Sc*_ura3 CP containing construct gave a much higher expression than constructs with the *An*_nirA CP.[17] The *nirA* gene encodes a transcriptional regulator mediating nitrate induction and is constitutively expressed at extremely low levels.[42] The *URA3* gene is commonly used as an auxotrophic marker in yeast and the *Sc_ura3* CP contains a TATA and a CCAAT box at around -100 bp relative to the start codon (see Supp. Figure S2, Table S1). In *S. cerevisiae*, the full-length *URA3* promoter is reported to be relatively weak.[43] The *An*_nirA CP contains none of the known CP elements and was even suggested to be a repressor element, despite functioning as a weak CP[17]. Nonetheless, control devices with an expression ranging from very low to high is needed for balancing pathways. Here, we have shown that the expression of our devices can be varied by changing the CP unit of the control device.

Benchmarking control devices against native promoters

Two of the control devices were benchmarked against native (full) promoters, the strong *pcbC* promoter, that is widely used for overexpression of genes in *P. chrysogenum,* and the less employed *pcbAB* promoter (Figure 6c). The expression of the control device containing 5xQUAS upstream of the *Pc*_pcbC CP was similar to the expression under *pcbC* and *pcbAB* during the first 30 h, after which the expression was somewhat lower than the expression under the native promoters (Figure 6c). In contrast, the expression of the control device containing 5xQUAS upstream of the *Pc*_pcbAB CP was at its peak (at 60 h) only about one-tenth of the expression of the native *pcbAB* promoter.

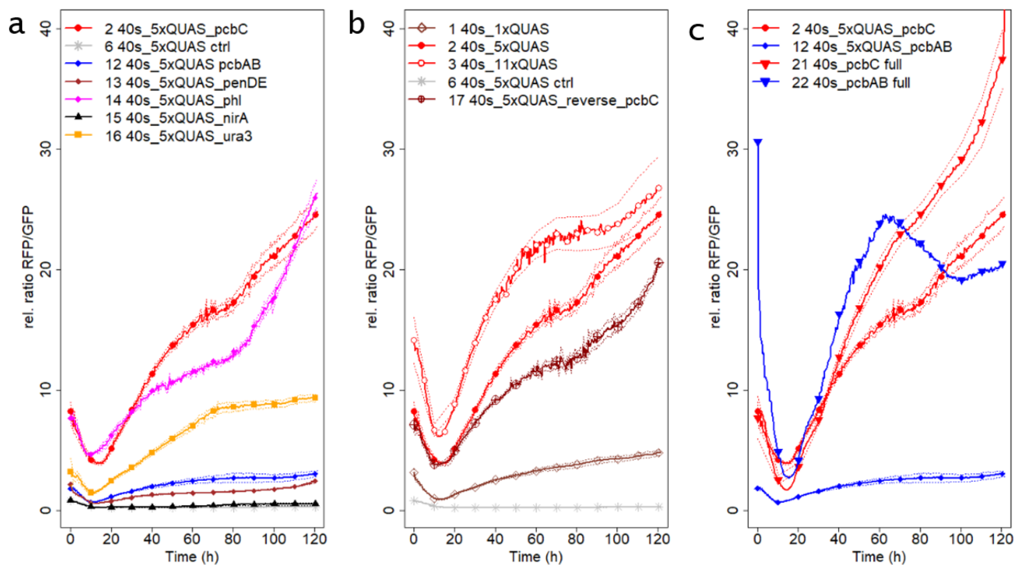


Figure 6. Development of RFP/GFP fluorescence over time during growth of selected *P. chrysogenum* strains. A) The strains contain control devices with different CPs. b) The strains express RFP under a synthetic promoter containing 1 (open diamonds), 5 (closed symbols, in 5'→3' direction; open with a plus in 3'→5' direction), 11 (open symbols) or no (stars) QUAS elements upstream of the *Pc*_pcbC CP. c) The strains express RFP under the full promoters of *pcbC* (red triangles down) or *pcbAB* (blue triangles down) or under a synthetic promoter containing 5xQUAS elements upstream of the *Pc*_pcbC (red circles) or *Pc*_pcbAB (blue diamonds) CPs. Solid lines indicate mean values for at least 3 independent cultures, the dashed lines show the standard error. The difference in expression of RFP of all strains expressing functional control devices, was significant ($p < 0.0001$) compared to the base strain (strain no. 2, 40s_5Q_pcbC).

The expression of RFP under the *pcbC* or *pcbAB* promoter was similar during the first 65 h, after which the expression per GFP or biomass of the construct with *pcbAB* declined. Notably, the biomass of the strain containing the *pcbAB* promoter decreased at the end of the cultivation, while the biomass of the strain containing the *pcbC* promoter remained constant or decreased only later. The biomass measured in the BioLector microwells showed variability at the end of the cultivations, thus the difference between the strength of the *pcbAB* and *pcbC* promoter may not be significant under different conditions. In a previous study, it was observed that the *pcbAB* promoter was constantly much stronger than *pcbC*[6]. In this study by Polli *et al.*[6], 10 bp upstream of the start codon was

lacking for both promoters, which may explain differences in expression.

The *pcbC* and *pcbAB* genes of *P. chrysogenum* face opposite directions and their intergenic region of around 1 kbp forms a bidirectional promoter. Both the *pcbAB* and *pcbC* genes are among the highest expressed[44] and the *pcbAB* and *pcbC* promoters were shown to be among the strongest tested to drive expression of a fluorescent protein[6]. In chemostat cultivation, the expression of *pcbAB and penDE* was reported to be approximately 80% or 40% of the expression of that of *pcbC*, respectively.[44] *Phl* is expressed at relatively low levels; the expression during glucose-limited chemostat cultivation was approximately 4% of that of *pcbC* in a high penicillin producing strain, containing 8 copies of the penicillin gene cluster.[44] Thus, there was no correlation between the reported native expression of the genes from which the CPs originate and the synthetic promoters containing the respective CPs. This is likely due to native regulation being disturbed in CPs. Still, the best performing control devices constructed showed a strength similar to the strongest promoters known for *Penicillium*.

Tuning expression by varying the QUAS element

The possibility to tune the expression levels is perhaps the most important feature of a control device. Modulation of expression by varying the number of UAS elements in the CP has been shown previously in various systems.[19,45–47] Therefore, constructs with one, five or eleven QUAS elements upstream of the *Pc*_pcbC CP were evaluated. As expected, the number of QUAS elements had a direct influence on the level of expression of RFP (Figure 6b). The promoter containing five QUAS binding sequences led to a final expression that was approximately 5-fold higher than a promoter with a single QUAS binding sequence. The expression under the construct with 11xQUAS binding sites was during the first 60 h about 50% higher compared to the 5xQUAS construct (Figure 6b), but leveled off after around 60 h, leading to a final expression similar to that of the construct with 5xQUAS. In line with these observations, several previous studies[19,45,47] report that the number of UAS elements influences the strength of expression, but the expression levels off or even decreases after a

certain number of repeats. It may be that the availability of transcription factors becomes limiting or that the increased amount of RNA cannot be translated into protein due to lack of available amino acids or energy. The observation that the expression in the construct with 5xQUAS was about 5 times the expression of the construct with 1xQUAS suggests that the short linker (2 bp) between the binding sites was not limiting the binding of the STF.

The bidirectional promoter of the *qa-1F* and *qa-1S* genes of the quinic acid cluster of *N. crassa* contains a common QUAS element.[29] This study confirms that the QUAS elements function in both directions (Figure 6b), which is highly applicable for construction of synthetic pathways. Bidirectional promoters are very common in SM clusters, but the expression of the bidirectional genes may vary.[10,44] When the 5xQUAS containing element was placed in the reverse direction (3'→5') upstream of the *Pc*_pcbC CP, the expression of RFP was identical during the first 25 h of growth, after which it was lower compared to the construct with the QUAS elements in the original direction. This amounted to about 80% of the expression of the construct with the QUAS elements in 5'→3' direction during the time interval of 40 - 120 h. The strength of the different variants of the QUAS elements (see Supp. Table S2; different repetitions of the GGRTAANNNNTTATCC sequence were designed to avoid spontaneous recombination), was not studied but may influence the overall strength of the control device and be more pronounced in one direction compared to the another. Quite some variability is seen in the QUAS elements of the native quinic acid pathway genes of *N. crassa*, leading to a large difference in affinity towards QF.[29] In line with this, Kiesenhofer *et al*.[48], showed that inverting repeats of cis-elements in the *T. reesei cbh1* promoter can be used to modulate expression. As our device containing 5 slightly varying repeats of the QUAS element showed an expression of five times the construct containing only one element it may be assumed that the difference in affinity was not greatly influenced by the variability of the sequence and that the expression of the device can be tuned by altering the number of the binding sites.

Regulation of the penicillin cluster using the control device

The penicillin cluster of DS54468 (1 copy) was placed under the regulation of the control device. Full-length promoters or CPs as well as different number of STF binding sequences (5x and 11x repeated) in constructs for expression of the penicillin synthesis genes were explored (Table 2, Figure 7). All strains contained a STF driven by P*40S* and a construct where the 5xQUAS element is put upstream of the *Pc*_penDE CP that drives *penDE*. Penicillin V production under synthetic regulation was successful in all strain variants and the Penicillin V titers achieved were dependent on the constructs used for expression of *pcbAB* and *pcbC* (Table 2), reaching levels also observed with the native promoter. After 5 days, all cultures had reached a biomass of ~15g/kg broth.

Table 2. Penicillin V production in shake flask cultures and characteristics of strains where the penicillin cluster was put under the control of the synthetic transcriptional factor. All strains were derived from DS54468.

Strain No.	Promoter for expression of *pcbAB*	QUAS elements of *pcbAB-pcbC* locus	QUAS elements of *penDE* locus	Penicillin V titer (g/L)*		
				after 3 days	after 5 days	after 7 days
DS54468	*Pc*_pcbAB	-	-	0.34±0.016	0.48±0.023	0.48±0.006
23	*Pc*_pcbAB CP	5xQUAS	5xQUAS	0.04±0.001	0.06±0.000	0.07±0.002
24	*Pc*_pcbAB CP	11xQUAS	5xQUAS	0.05±0.002	0.08±0.001	0.14±0.005
25	*Pc*_pcbAB full	5xQUAS	5xQUAS	0.14±0.002	0.26±0.002	0.33±0.011
26	*Pc*_pcbAB full	11xQUAS	5xQUAS	0.20±0.003	0.38±0.003	0.49±0.006

*mean ± S.E.M of 3 biological replicates, with 3 technical replicates each.

The characterization of the control devices in microbioreactors, proved to give excellent indications for how the devices function in the production strains and conditions. In strains where only the respective CPs drive *pcbAB* and *pcbC* (strains 23 and 24), less Penicillin V was produced compared to the parental strain (DS54468), likely due to the *Pc*_pcbAB CP being a very weak CP (Figure 6a). The *Pc*_penDE CP was also shown to be rather poor, only about 13 % compared to the device containing *Pc*_pcbC CP (Figure 5a). However, the transcription of *penDE* in native penicillin production strains is also much lower than expression of *pcbAB* or *pcbC.*[44]

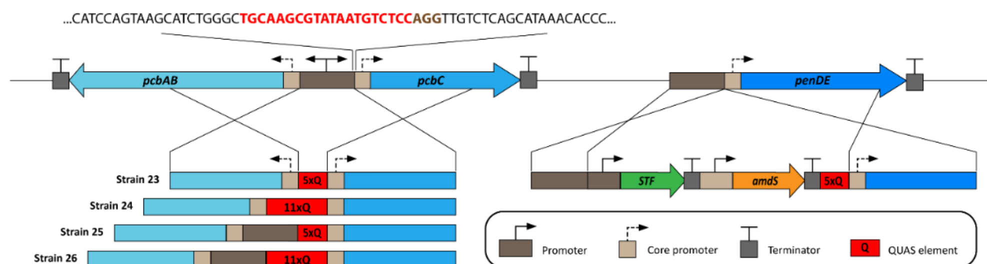


Figure 7. Schematic representation of CRISPR/Cas9 mediated co-transformation of the synthetic control device into the penicillin cluster. The marker-free donor DNA carried QUAS elements with flanking regions for homologous recombination at the *pcbAB*/*pcbC* locus. The *amdS* marker carrying integration cassette delivers the STF and a 5xQUAS element upstream of the core promoter of *penDE* gene.

During the whole experiment, 11xQUAS carrying constructs overperformed their 5xQUAS counterparts both in *Pc*_pcbAB CP (strain 23, 24) and *Pc*_pcbAB full promoter (strain 25, 26) utilizing setups. In strain 25 with a full promoter driving *pcbAB* and the synthetic control devices with 5xQUAS and respective CPs upstream of *pcbC* and *penDE*, the production was ~70% of that of the parental strain (DS54468). The final Penicillin V titer of strain 26 with 11xQUAS was ~150% of the titer produced with strain 25 containing 5xQUAS and reached the production levels of the parental strain (DS54468). The use of constructs with a high number of QUASs combined with CPs that would allow a higher and faster expression of all the penicillin cluster genes may be expected to lead to strains with increased penicillin production rate. The scalability demonstrated by fluorescence reporters was verified by synthetically controlling the production of penicillin.

Conclusions

Filamentous fungi are attracting increasing interest as biotechnological production hosts, but efficient genetic tools for exploitation were lacking. Therefore, we successfully developed a modular, synthetic control device for *P. chrysogenum* and demonstrated its function through regulation of the penicillin cluster. The strength of the control device was altered by altering the expression of the synthetic transcription factor (STF), the core promoter downstream the QF

Upstream activating sequence (QUAS), or the amount of QUAS elements, leading to an expression ranging from hardly detectable to an expression similar to that of highest expressed native genes. A gene cluster may demand an expression tuned individually for each gene, which is a great advantage provided by this system. We anticipate that these well-characterized and robustly performing control devices can be highly useful tools in the development of filamentous fungi as production hosts.

Methods

Fungal strains and culture conditions

P. chrysogenum DS68530 (ΔPenicillin-cluster, Δ*hdfA*, derived from DS17690)[33,49] and DS54468 (1xPenicillin-cluster, Δ*hdfB*, derived from DS47274)[50] strains were kindly provided by Centrient Pharmaceuticals B.V., former DSM Sinochem Pharmaceuticals, the Netherlands.

Fungal strains were purified and grown on solid complex or transformant selection medium with 0.1% acetamide as a sole nitrogen source.[6,51] Mycelium from the complex medium was collected for long term storage of strains on rice grains or for microscopy analysis. Spores (immobilized on 25 rice grains) or biomass grown on solid complex medium for 2-3 days until sporulation, were used to inoculate 25 or 10 ml (using spores or biomass, respectively) SM production medium.[6] Cultures were incubated for 42-50 h in a rotary incubator at 200 rpm at 25°C.

For BioLector analysis and analysis of growth in FlowerPlate (MTP-48-B) wells, this pre-grown mycelium was diluted 8 times in fresh SM production medium. The 1 ml cultures were grown in the BioLector microbioreactor system (M2Plabs, Germany), shaking at 800 rpm at 25°C. In the BioLector, biomass was measured via scattered light at 620 nm excitation without an emission filter. The fluorescence of GFP-NLS and DsRed-SKL (for simplicity referred to as GFP and RFP in the text) was measured every 30 min with 486/589 nm excitation filter and 510/610 nm emission filter, respectively. In contrast to our previous work[6], the wavelength used for measuring RFP fluorescence was optimized for DsRed.

For measurements of carbon consumption, the experiment was disrupted after 24h and contest of wells was analyzed by HPLC (Shimadzu, Japan) using an HPX-87H column (Shimadzu, Japan), 0.005 M H_2SO_4 with a flow rate of 0.6ml/min on 65°C. All experiments were conducted in at least technical 4 replicates, of at least 2 different biological replicates. The data obtained from the BioLector experiments were analyzed using the TIBCO Spotfire Software (TIBCO Software Inc., USA) and presented using RStudio and the Plotrix package.

For penicillin fermentation, strains with STF and the Q-system regulatory elements integrated in the penicillin cluster were grown in YGG medium for 24 h, after which the cultures were diluted 8 times into penicillin production medium supplemented with 2.5 g/L phenoxyacetic acid, mediums prepared as described previously.[52] Supernatant samples for HPLC analysis were taken after 3, 5 and 7 days and extracellular Penicillin V titers were determined by UHPLC (Shimadzu Nexera UHPLC, Japan) using a Shim-pack XR-ODS 2.2 (75mm L. x 3mm i.d.) column operating at 40°C according to Weber *et al*.[52]

Construction of expression cassettes for control devices

PCR amplifications were conducted using KAPA HiFi HotStart ReadyMix (Roche Diagnostic, CH) or Phusion High-Fidelity DNA Master Mix (Thermo Fisher Scientific, USA), for primers see Supp. Table S5. The MoClo modular cloning system[53] was employed for construction of all expression cassettes (Table 1, 2, Supp. Figure S1, for more details see Supp. Table S3-4, Note S1). Flanking regions of approximately 800 bp were designed for integration of the expression cassettes at the locus of the deleted penicillin cluster of DS68530 by *in vivo* homologous recombination. Internal BsaI, BpiI and in most cases also DraIII recognition sites of the DNA elements were removed during the cloning. A modified protocol using the FastDigest versions (Thermo Fisher Scientific, USA) of the BsaI and BpiI restriction enzymes were used with an initial 10 minutes digestion, 20-50 cycles of digestion and ligation (37°C for 2 min, 16°C for 5 min), followed by a final digestion step and a heat inactivation step, was used for most assemblies, instead of the standard MoClo protocol.

The endogenous elements for the expression cassettes constructed in this study, were amplified by PCR from genomic DNA of *P. chrysogenum* DS54468. The *amdS* selection cassette used was described previously[54]. The 138 bp Sc_*ura3* CP amplified from genomic DNA of *S. cerevisiae* CEN.PK, is slightly longer compared to the version used by Pachlinger *et al.*[17] The DsRed-SKL gene was amplified from the pJAK109 plasmid[54] while the promoter of *A. nidulans* ribosomal protein S8 (AN0465.2, referred to as *40S*) was amplified from pDSM-JAK108[39]. The *gndA* promoter (Sequence ID: AM270223.1 32820 to 32040) from *A. niger CBS 513.88* was ordered as a synthetic DNA from IDT. The *GFP* was amplified from the pSpCas9-2A-GFP plasmid, kindly provided by Feng Zhang via Addgene (Plasmid #48138[55]). The pAC-7-QFBDAD plasmid, used for amplification of the QF DBD was kindly provided by Christopher Potter, via Addgene (Plasmid #46096[25]). The plasmid pVG2.2 used as a template for the VP16 activation domain was a gift from Vera Meyer.[12] The 94 bp long *An*_NirA CP (identical to the sequence used by Pachlinger *et al*.[17]) as well as the cassettes containing 1 or 5xQUAS sequences, were ordered as oligos that were annealed before assembly to level 0 vectors, the initial building blocks used in the MoClo system. The repetitions of the QUAS elements were designed to contain some variability as the genetic stability of *P. chrysogenum* strains was an initial concern. The 11xQUAS carrying plasmid was constructed with the assembly of three units of annealed oligos (see Supp. Table S2). The design was for creating a 15xQUAS containing part, but this was proven to be difficult for *E. coli* to assemble, as some QUAS sequences were looped out during the construction. The sequence of the actual 11xQUAS part constructed can be found in Supp. Table S2.

Construction of a Q-system controlled penicillin production strain

The 5 or 11xQUAS elements were inserted in the intergenic region between the *pcbAB* and *pcbC* genes (leaving 200 bp CPs upstream each gene) of the penicillin cluster of DS54468 (Figure 6, Strains 23, 24) using co-transformation and the CRISPR/Cas9 technology described previously.[54,56] Strains where the QUAS elements were inserted 200 bp upstream the *pcbC* gene but leaving

the *pcbAB* promoter intact were created in a similar manner (Figure 7, Strains 25, 26). The integration of the marker-free dDNA was facilitated with *in vitro* preassembled CRISPR/Cas9 ribonucleoproteins where the sgRNA was targeting the TGCAAGCGTATAATGTCTCCAGG sequence at the boundary between the promoter of *pcbAB* and the CP of *pcbC*.

The dDNA for integrating the 5xQUAS elements upstream of the CP of *penDE* also contained the STF and an *amdS* marker (YN2_71, Supp. Table S4). One µg plasmid containing ~1 kbp homologous 3' and 5' flanking regions for integration upstream to *penDE* was digested with DraIII before co-transformation with marker-free DNA. 5 µg marker-free dDNA cassette carrying plasmid (YN1_81, YN1_82, YN1_77, YN1_80 for strains 23, 24, 25 and 26 respectively, see Supp. Table S4) were digested with KspAI and PaeI leaving ~1-2.5 kbp homologous flanking regions around the QUAS elements for creating *Pc*_pcbAB CP or *Pc*_pcbAB full promoter strains (Figure 7). All dDNA cassettes were build using the MoClo system.[53,57] Correct clones were selected using colony PCR and confirmed by sequencing. The strains were purified through 3 rounds of sporulation before liquid culture cultivation.

Copy number determination by qPCR analysis

Copy numbers of genes and constructs were determined using the MiniOpticon™ system (Bio-Rad, USA) for analyzing gDNA isolated as described before.[49] SensiMix™ SYBR mix HI-ROX (Bioline, UK) was used as a master mix for qPCR with 0.4 µM primers and 10 ng gDNA in a 25 µL reaction volume. Data were analyzed using the BioRad CFX manager software in which the C(t) values were determined automatically by regression.[49] Copy numbers were calculated from duplicate experiments with three technical replicates, using the γ-actin gene (Pc20g11630) as a control for normalization.[49] The efficiency of the primers used for the copy number determination was assessed through the use of four dilutions of gDNA. Primers used for *pcbC*, *penDE*, and *STF* copy number identification (see Supp. Figure S3) on strains 23,24,25,26 are listed in Supp Table S5. The γ-actin, *pcbC*, *PenDE* and STF showed efficiencies of 100.17% (R^2 = 1.000), 102.86%

(R^2 = 0.993), 96.38% (R^2 = 0.999) and 97.87% (R^2 = 0.998), respectively. *P. chrysogenum* DS54468 and DS68530 strains were used as controls containing zero copies of STF, and 1 or 0 copies of the penicillin gene cluster, respectively.

Fungal transformations and analysis of transformants

Transformations of *P. chrysogenum* were performed as described previously[51], using about 1.5 µg of digested plasmid(s) for each transformation. The expression cassettes were digested with MreI or DraIII, that cut twice in the backbone of the MoClo vectors. For some transformations, the protoplasts were cryopreserved, based on the method described for *U. maydis.*[58] After the final washing step of the protoplast formation, the protoplasts were suspended in STC medium and diluted twice in cryopreservation medium; 20% PVP-40 (Polyvinylpyrrolidone 40 $(C_6H_9NO)_{40}$) in STC buffer (1.2 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5), and were frozen in cryo tubes using a slow cooling rate in a Mr. Frosty freezing container. To confirm the integration of the cassettes at the correct locus, colony PCR was performed using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific, USA) or with standard PCR reactions using DNA extracted from the cells using Lysing Enzymes from *Trichoderma harzianum* (Sigma-Aldrich, UK). In addition, PCR products of selected transformants were sequenced. All strains that were analyzed by sequencing of the QUAS and the CP region contained all designed QUAS repetitions, thus no strain instability due to repetitive elements was observed. For some constructs, we however observed ectopic recombination, regardless of the strains being of deficient in non-homologous end-joining (*ΔhdfA/hdfB* background); these transformants were dismissed from the core study.

Fluorescence microscopy

Transformants were examined using fluorescence microscopy after 4 days of growth on acetamide solid medium. A small amount of hyphae was taken from the peripheral zone of the colonies and suspended in phosphate-buffered saline (58 mM Na2HPO4; 17 mM NaH2PO4; 68 mM NaCl, pH 7.3). Samples analyzed

for nuclear localization were stained with 4'-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, UK) at 1 µg/ml in PBS buffer for 20 min. Samples were examined with Nikon Ti-E microscope (Nikon Instruments, Tokyo, Japan) equipped with Hamamatsu Orca Flash 4.0 camera with 100x objective, numerical aperture: 1.45. refractive index: 1.515. Pictures were taken using phase contrast, DAPI, FITC (GFP) and TRITC (RFP) filters. Strains no. 2, 4, 5, 10, 11, 15-20 were examined with fluorescence microscopy.

List of abbreviations

SM, Secondary metabolites; TF, Transcription factor; DBD, DNA-binding domain; AD, Activation domain; UAS; Upstream activating sequence; STF, Synthetic transcription factor; QUAS, QF Upstream activating sequence

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Authors' contributions

Y.N, A.J.M.D. and R.A.L.B. designed the experiments; Y.N and L.M. performed the experiments, with the help of Z.B. Y.N and L.M. wrote the manuscript with the improvements from the other authors. All authors read and approved the final manuscript.

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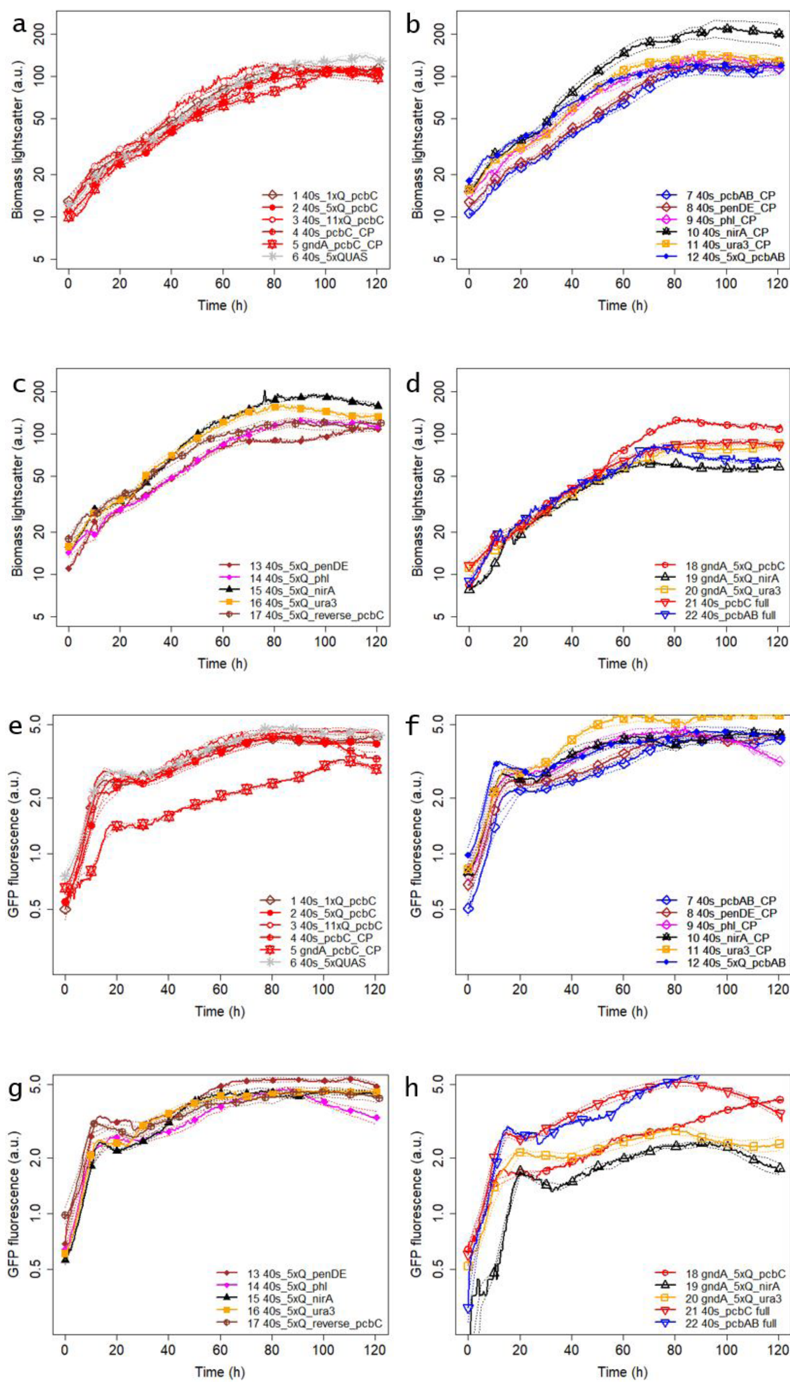
Supplementary information



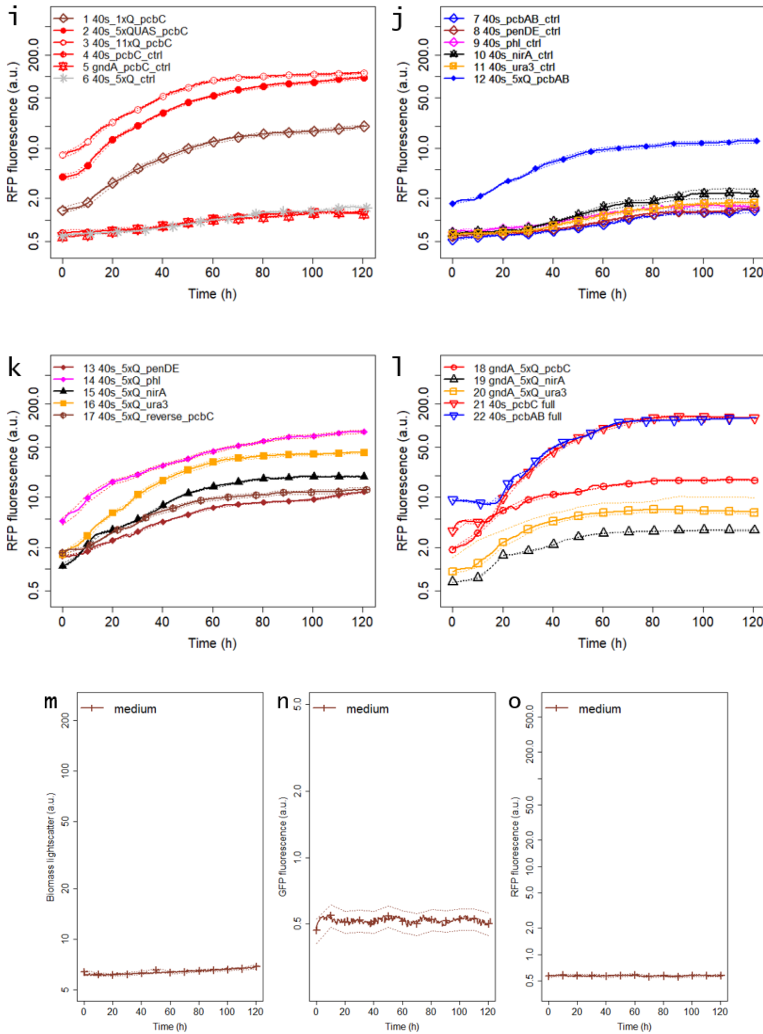


Figure S1: Development of biomass (a-d), GFP fluorescence (e-h), GFP and RFP fluorescence (i-l) over time of all strains, described in Table 1, shown on logarithmic scales. Strain numbers and core promoters of strains are marked in figure legends. The promoter used for expression of the STF was P*40S* or P*gndA* (marked in legend). Background light scatter and fluorescence of medium shown in figure m-o, using same scales as in the corresponding figures showing data of strains (a-l). Solid lines indicate mean values for at least 3 independent cultures, the dashed lines show the standard error.

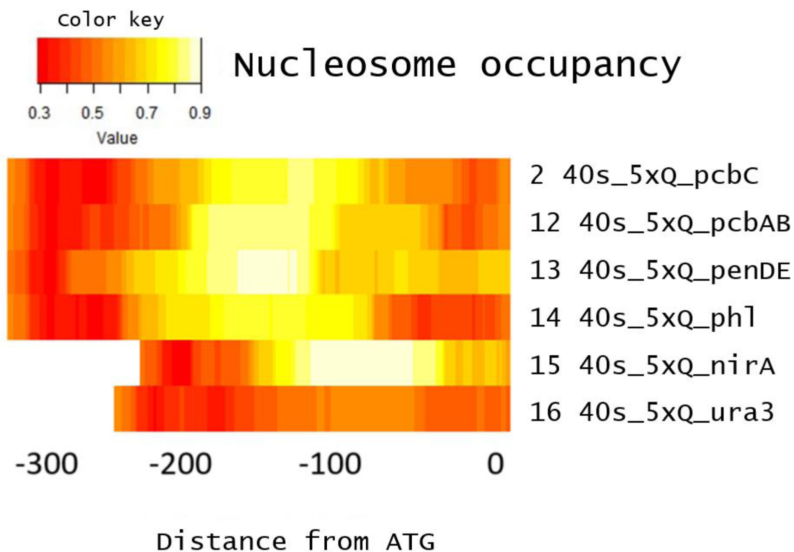


Figure S2. Nucleosome occupancy profile heatmap of the synthetic promoters, consisting of the CPs fused to 5xQUAS. The nucleosome occupancy was calculated according to Kaplan et al[1]. The lower nuclear occupancy of the Sc_ura3 CP may explain the relatively high expression of the synthetic promoter containing this CP (Figure 5a), despite an apparent difference in this CP in terms of GC content (Table S1). AT rich sequences have been reported to be associated with low nucleosome affinity and high promoter activity[2].

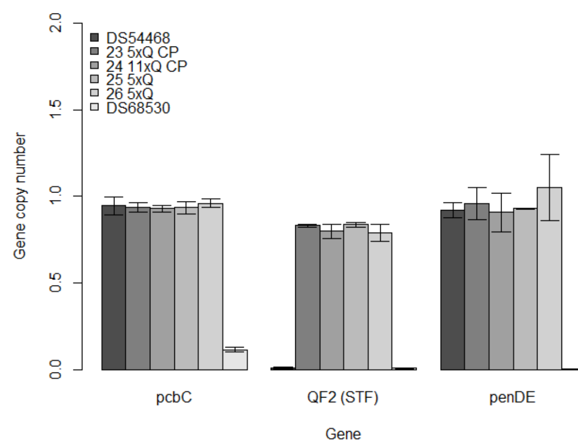


Figure S3. Strain verification through qPCR. Quantification of the copy number of the integrated donor DNA and two penicillin cluster genes (*pcbC* and *penDE*) in *P. chrysogenum* strains. Strains DS54468 (1xPen-cluster) and DS68530 (ΔPen, no penicillin cluster) were used as controls.

Table S1: Characteristics of core promoters (CPs) used in the study. Numbers indicate position of the CP element in relation to the start of the gene (ATG).

CP	Size	GC cont.	TSS	TATA	CCAAT	INR
*Sc*_ura3	94	40%	Multiple, -119 − -60	-92, -90	-98	-99, -101
*An*_nirA	138	54%	Unknown	-	-	-
*Pc*_pcbC	200	51%	-15	-194	-	-11, -107
*Pc*_pcbAB	200	50%	-106	-94,-131	-12, -18, -111, -149	-94, -131, -164
*Pc*_penDE	200	55%	-73	-104	-107, -121	104, -122, -194
*Pc*_phl	200	50%	Unknown	-21	-	-4, -19, -48, -122

TSS = Transcription starting site, according to Šmidák et al.[3] or Losson et al.[4]. INR element = Initiator element; YYANWYY

Table S2: Sequence of synthetic fragments used

ID	Description	Sequence (5' -> 3')
A	P*gndA*	TTGAAGACATGGAGTCTTGCGTTACGGGCGTATTTTGCTGCGGCCGGTGGTGCCCCTCCATGCCCCGCCATC TTTCAAAGCTCCTGGCGACGCCGTCATCTCCGAACATTCTCCCCCCAAAGGAATCAATTGGCAATTGGAGTC TAGTAAAGTGGTGTTTGTCATCAGTAAGGAGTTGGTGAAACTACAATCTTCCATCATGAAGAGAAGGGATATT TTTGGGGTTGTATTTTACGATGAAGGTACTGGAAATGGTGGGGGTTTTTATAGCAGTAGACAGTCAGTCAGT AAGTAGTATGCTTGTTGTATTACCCAAACCAGATCAATCCAAAGAAAGCCTGACAGACAGCCATCAATAGATA CTACTTCGTACTATAGTTACCCACCTAACCATATTACTCAAAAAGCATCTATCTATCCGCGGGCTTCCATGCATG TCCCGGTAGCAAACTCCTCCCACCGGTGTAGTACTCTTTGGTTAGTAGTCTTGTTCACCGGAGGACTCTGCTC CTCTCCTGCTCAGGTGCTGCCCCGCCCTCCGTCCCACCATGACGGAAGAGATGCTCCGTAAGCCGTCCAGTT GCAACGAATCCTGCTCTGACATCTTCGAACGCCTTCTCCCTTTCGCTCGCTTCTCTGCCTCTTTCCTCTCTTCCC TTTCCTTCCCCTCCAAACTAAACCTTCCTCCTTTTCTCCATCATCCTCTAGGCAGTTGGTTCTTCCTGACTGTACA TATATCCACCACCTCCCCCCTCTATTCTTCCACCTCTTCCATATCTCCTTCTCCAGAGTTCATACCCCCCACAATG ATGTCTTCTT
B	1xQUAS	TTGAAGACAAGGAGGGGTAATCGCTTATCCCCATAAGTCTTCTT
C	5xQUAS	TTGAAGACAAGGAGGGGTAATCGATTATCCTCGGATAAACACTTATCCCAGGGTAA TCACTTATCCCTGGATAAACAATTATCCTCGGGTAATCGCTTATCCCCATAAGTCTTCTT
D	15xQUAS A	GTGAAGACTAGGAGGGGTAATCGATTATCCTCGGATAAACACTTATCCCAGGGTA ATCACTTATCCCTGGATAAACAATTATCCTCGGGTAATCGCTTATCCTACGTTGTCTTCAC
E	15xQUAS B	GTGAAGACAATACGGGGTAATCGATTATCCTCGGATAAACACTTATCCCAGGGTAATC ACTTATCCCTGGATAAACAATTATCCTCGGGTAATCGCTTATCCTTGCTTGTCTTCAC
F	15xQUAS A	GTGAAGACAATTGCGGGTAATCGATTATCCTCGGATAAACACTTATCCCAGGGTAA TCACTTATCCCTGGATAAACAATTATCCTCGGGTAATCGCTTATCCCCATTTGTCTTCAC
G*	11xQUAS	TTGAAGACAAGGAGGGGTAATCGATTATCCTCGGATAAACACTTATCCCAGGGTAATCACTTATCCCT GGATAAACAATTATCCTCGGGTAATCGCTTATCCTACGGGGTAATCGCTTATCCTTGCGGGTAATCGC TTATCCTCGGATAAACAATTATCCCAGGGTAATCACTTATCCCTGGATAAACAATTATCCTCGGGTA ATCGCTTATCCCCATAAGTCTTCTT
H	nirA CP	TGAAGACAACCATCAGGGAAACACGCCGAGCGTCCTCCTCCGATAAGCATGCGCTGTCTT GGTCTGTCGCTGTCACCGAACATATCTGTCTGGGCTTGATTTACCATAATGAAGTCTTCT
I	nirA CP ctrl	TGAAGACAAGGAGCAGGGAAACACGCCGAGCGTCCTCCTCCGATAAGCATGCGCTGTCT TGGTCTGTCGCTGTCACCGAACATATCTGTCTGGGCTTGATTTACCATAATGAAGTCTTCT

*=verified by sequencing after cloning event

Table S3: List of primers used for amplification of PCR products for cloning

ID	Description	Primer sequences (5'->3')
1	Pen 5'flank A	F:ACAGCGGAAGACAAGGAGCCTGCAGGATGGGCCTCCACAACCCTGCC R:TGAAGACAACGAATCTTCTATTCAATCTGATAAC
2	Pen 5'flank B	F:TGAAGACAATTCGGTGATGCAGCAAATAGCGA R:TGAAGACAATGAGGACAGGATATCACGCGTTACC
3	Pen 5'flank C	F:TGAAGACACCTCAGTCTTAAGACTTCTCACCTA R:ACAGCGGAAGACAAAGCGCTACCGTTTGTACCATCTGA
4	P*40S* A	F:TTGAAGACAAGGAGGAGTTATAGACGGTCCGGCATAGG R:TTGAAGACAAGGTGTCGATCGGACGTATTGTCCAAG
5	P*40S* B	F:TTGAAGACAACACCAAAGCAATCTGGTACATCACCC R:TTGAAGACAAGGTATCCTCCACAGACTCCTTGAGCC
6	P*40S* C	F:TTGAAGACAATACCTGATATAAGAATTGAGAGTTATACTCCGG R:TTGAAGACAACATTGTTTGCTGTCTATGTGGGGGACTG
7	QF DBD	F:TTGAAGACATAATGCCACCCAAGCGCAAAAC R:TTGAAGACAAACCTGAGGAGGCGGGTAATGCTCTTATTG
8	VP16 AD	F:TTGAAGACAAAGGTTTGAAAGCGGCGGGCCGG R:TTGAAGACAACGAACCCGGGGAGCATGTCAAGG
9	QF AD	F:TTGAAGACAAAGGTTTCGTCAGTTGGAGCTCCCTCCTA R:TTGAAGACAACGAACTGTTCGTATGTATTAATGTCGGAGA
10	*eGFP*-NLS	F:TTGAAGACAATTCGTTATGGTGAGCAAGGGCGAGGAGC R:TTGAAGACAAAAGCTTAGACCTTCCGCTTCTTCTTTGGCTTGTACAGCTCGTCCATGCC
11	t*tif35*	F:ACAGCGGAAGACAAGCTTACTTCTTTATCGGTTCTCTCTTACGAC R:ACAGCGGAAGACAAAGCGGTGCTTGGGATGTTCCATGGTAGCTGTG
12	P*gpdA*	F:ACAGCGGAAGACAAGGAGTGGATCCCCCGGGCTGCAGG R:GAAGACTTCATTGTGATGTCTGCTCAAGCGGG
13	*amdS;* A	F:GAAGACTTAATGCCTCAATCCTGGGAAGAAC R:TGAAGACAAGTCATCCGCAGGCAGCGTCTG
14	*amdS:* B	F:TGAAGACAATGACAGCGTTATTGATTTCCCAAAGAAATCG R:TGAAGACAAGGTGTCTTGTGCTTTGCGTAGTATTCA
15	*amdS;* C	F:TGAAGACAACACCCGTTGGTCCACTCCAT R:TGAAGACAAGAAAACGGCACCGGCTTTGCGG
16	*amdS;* D	F:TGAAGACAATTTCTACGTCAAGACCTCTGTCCCG R:ACAGCGGAAGACAACATTACTTCATCAGTGACTGCCCGTCTCGTATATAGTATAAAA
17	*amdS;* E	F:TTGAAGACAAAATGTTAGACCTCCGCCTCTTCAC R:TTGAAGACAAGGTATGACCGGCTTTGGCGAGTGCG
18	*amdS;* F	F:TTGAAGACAATACCGTGACCCCGTGGACGC R:GAAGACAAAAGCCTATGGAGTCACCACATTTCC
19	t*amdS*	F:ACAGCGGAAGACAAGCTTCTAATAAGTGTCAGATAGCAAT R:ACAGCGGAAGACAAAGCGTACCGCTCGTACCATGGGTT
20	*ppcbC;* A	F:ACAGCGGAAGACAAGGAGCTGCATTGGTCTGCCATTGCAG R:TGAAGACAAAGATTAGTAAGTACTTATCATTACCGTG
21	*ppcbC;* B	F:TGAAGACAAATCTTCGAGCGGGGGAGTGTT R:ACAGCGGAAGACAACATTGGTGTCTAGAAAAATAATGGTGAA

ID	Description	Primer sequences (5'->3')
22	p*pcbAB;* A	F:TTGAAGACAAGGAGGTGCCTTACTGGATGGGGCC R:TTGAAGACAAAGTGCTTCGAGCGGGGGAGTG
23	p*pcbAB;* B	F:TTGAAGACAACACTAGTAAGTACTTATCATTACCGTGCCAG R:TTGAAGACAACATTGTCTGTCAATGACCAATAATTGGTAGGG
24	*Pc*_pcbAB CP	F:TTGAAGACAACCATATCTTGTCTGCGGGCAGTG R:TTGAAGACAACATTGTCTGTCAATGACCAATAATTGGTAGGG
25	*Pc*_pcbAB CP ctrl	F:TTGAAGACAAGGAGATCTTGTCTGCGGGCAGTG R:TTGAAGACAACATTGTCTGTCAATGACCAATAATTGGTAGGG
26	*Sc*_ura3 CP	F:TTGAAGACAACCATCAGAAGGAAGAACGAAGGAAGGAG R:TTGAAGACAACATTGATTTATCTTCGTTTCCTGCAGGTTTTTG
27	*Pc*_pcbC CP	F:TTGAAGACAACCATCGTATAATGTCTCCAGGTTGTCTCAGC R:TTGAAGACAACATTGGTGTCTAGAAAAATAATGGTGAAAACTTG
28	*Sc*_ura3 CP ctrl	F:TTGAAGACAAGGAGCAGAAGGAAGAACGAAGGAAGGA R:TTGAAGACAACATTGATTTATCTTCGTTTCCTGCAGGTTTTTG
29	*Pc*_pcbC CP ctrl	F:TTGAAGACAAGGAGCGTATAATGTCTCCAGGTTGTCTCAGC R:TTGAAGACAACATTGGTGTCTAGAAAAATAATGGTGAAAACTTG
30	*Pc*_phl CP	F:TTGAAGACAACCATCGTCACTTGTCATAAATCTCCCCTCA R:TTGAAGACAACATTTTTCCCAAATCCTCGGGGTAATATAGGG
31	*Pc*_phl CP ctrl	F:TTGAAGACAAGGAGCGTCACTTGTCATAAATCTCCCCTCA R:TTGAAGACAACATTTTTCCCAAATCCTCGGGGTAATATAGGG
32	*Pc*_penDE CP	F:TTGAAGACAACCATAGACTAGGCGGATGCAGCA R:TTGAAGACAACATTTTCTGCTGCGGGTCGGAAG
33	*Pc*_penDE CP ctrl	F:TTGAAGACAAGGAGAGACTAGGCGGATGCAGCA R:TTGAAGACAACATTTTCTGCTGCGGGTCGGAAG
34	*DsRe*d-SKL; A	F:TTGAAGACAAAATGGCCTCCTCCGAGGACGTCA R:TGAAGACAAAGTTTTCTTCTGCATTACGGGGCC
35	*DsRed*-SKL; B	F:TGAAGACAAAACTATGGGCTGGGAGGCCT R:TTGAAGACAAAAGCTTACAGCTTCGACTTGTACAATTCG
36	t*act1*	F:ACAGCGGAAGACAAGCTTGTGCTTCTAAGGTATGAGTCGCA R:ACAGCGGAAGACAAAGCGCGCAGGGTTTGAGAACTCCGATCT
37	Pen3'flank; A	F:ACAGCGGAAGACAAGGAGACTTTAGACATACCTCTCGT R:TGAAGACAAGAATACGTCATACTTATTCTCTGA
38	Pen3'flank: B	F:TGAAGACATATTCTTGGCAATGTTTAAGCTTG R:ACAGCGGAAGACAAAGCGCCTGCAGGCTATCCGATATGCCGTCTGC
39	*Pc_pcbC* CP with *pcbC;* A	F:TTGGTCTCAGGAGTAGACCTGGCTGACGGAGA R:TTGGTCTCACTCCCGTATAATGTCTCCAGGTTGTCTCAGC
40	p*pcbAB* with *pcbAB* flank; A	F:TTGGTCTCACCATGCTTGCAGCCCAGATGCTTAC R:TTGGTCTCAAGCGCCAGACTCGTGTCCTTACGGGTCGAC
41	*Pc*_pcbC CP with *pcbC*; B	F:TTGGTCTCAGGAGCAGCTCACTACCACGCAAATCT R:TTGGTCTCACTCCCGTATAATGTCTCCAGGTTGTCTCAGC
42	p*pcbAB* with *pcbAB* flank; B	F:TTGGTCTCACCATGCTTGCAGCCCAGATGCTTAC R:TTGGTCTCAAGCGGACAGTCGGAGAAACGCAGAG

ID	Description	Primer sequences (5'->3')
43	p*pcbAB* with *pcbAB* flank; C	F:TTGGTCTCACCATATCTTGTCTGCGGGCAGTG R:TTGGTCTCAAGCGGACAGTCGGAGAAACGCAGAG
44	5'flank *penDE;* A	F:TTGGTCTCAAGCGTGCCGAGGAGCTGGATTGAG R:TTGGTCTCAATCCCAAATCCGAGGGTAATGCAG
45	5'flank *penDE;* B	F:AAGGTCTCAGGATGACAGTGTAATCCGCCGCAAG R:TTGGTCTCAGGAGGACTGAACCTCTTCGAGATAACAAGATTTTTC
46	3'flank *penDE;* A	F:TTGGTCTCAAATGCTTCACATCCTCTGTCAAGG R:TTGGTCTCACGTTTTCTTCGTTTTCCCTCGGATGAGATC
47	3'flank *penDE;* B	F:TTGGTCTCAAACGGACGAAGAGCTTAAACAGG R:TTGGTCTCAAGCGGACCCTGAAGGTGAAGGGC

Table S4: MoClo plasmid construction. Plasmids were constructed using PCR products, synthetic DNA fragments and MoClo compatible plasmids. Description of DNA parts cloned into MoClo vectors listed under Part IDs, described in Table S2-3.

Plasmid	Description	Part IDs	MoClo vector
pZB0_1	5' flanking region for integration at the deleted penicillin cluster of DS68530	1, 2, 3	pICH41331
pZB0_21	promoter of AN0465; *40S* ribosomal protein S8e	4, 5, 6	pICH41295
pFG0_1	promoter of An11g02040; *gndA*	A	pICH41295
pLM0_1	DNA binding domain of QF TF	7	pICH41258
pLM0_5	Activator domain of VP16	8	pAGM1299
pLM0_2	Activator domain of QF TF	9	pAGM1299
pYN0_29	*eGFP-NLS*	10	pAGM1301
pYN0_10	terminator of PC22g19890; *TIF35*	11	pICH41276
pLM0_12	promoter of AN8041; *gpdA*	12	pICH41295
pLM0_11	AN8777*; amd*S	13, 14, 15, 16, 17, 18	pICH41308
pZB0_20	terminator of AN8777; *amdS*	19	pICH41276
pYN0_58	1xQUAS	B	pAGM1251
pLM0_3	5xQUAS	C	pAGM1251
pLM0_8	11xQUAS	G	pAGM1251
pZB_0_23	full length promoter of Pc21g21380; *pcbC*	20, 21	pICH41295
pYN0_85	full length promoter of Pc21g21390; *pcbAB*	22, 23	pICH47761
pYN0_63	CP of Pc21g21390; *pcbAB*	24	pAGM1276
pYN0_64	CP of Pc21g21390; *pcbAB* (No QUAS control)	25	pICH41295
pYN0_21	CP of YEL021W; *URA3*	26	pAGM1276
pYN0_22	CP of AN0098; *nirA*	H	pAGM1276
pYN0_23	CP of Pc21g21380; *pcbC*	27	pAGM1276
pYN0_24	CP of YEL021W; *URA3* (No QUAS control)	28	pICH41295
pYN0_25	CP of AN0098; *nirA* (No QUAS control)	I	pICH41295
pYN0_26	CP of Pc21g2138; *pcbC* (No QUAS control)	29	pICH41295
pYN0_67	CP of Pc22g14900; *phl*	30	pAGM1276
pYN0_68	CP of Pc22g14900; *phl* (No QUAS control)	31	pICH41295

Plasmid	Description	Part IDs	MoClo vector
pYN0_65	CP of Pc21g21370; *PenDE*	32	pAGM1276
pYN0_66	CP of Pc21g21370; *PenDE* (No QUAS control)	33	pAGM1251
pZB0_26	*DsRed-SKL*	34, 35	pICH41308
pYN0_9	terminator of AN6542; *Act1*	36	pICH41276
pZB0_2	3' flanking region for integration at the deleted penicillin cluster of DS68530	37, 38	pICH41331
pYN1_77	marker free donor DNA for 5xQUAS regulated CP of *PcbC* and p*pcbAB,* for making Strain 25	pLM0_3, 39, 40	pICH47761
pYN1_80	marker free donor DNA for 11xQUAS regulated CP of *PcbC* and p*pcbAB,* for making Strain 26	pLM0_8, 41, 42	pICH47761
pYN1_81	marker free donor DNA for 5xQUAS regulated CP of *PcbC* and CP of *pcbAB,* for making Strain 23	pLM0_3, 41, 42	pICH47761
pYN1_82	marker free donor DNA for 11xQUAS regulated CP of *PcbC* and CP of *pcbAB,* for making Strain 24	pLM0_8, 41, 43	pICH47761
pYN1_78	5' flanking region for integration at *PenDE* of DS68530	44, 45	pICH47732
pYN1_79	3' flanking region for integration at *penDE* of DS68530	pLM0_5, pYN0_65, 46, 47	pICH47761
pYN2_71	*amdS* marker containing donor DNA for integrating the STF and the 5xQUAS regulated CP of *PenDE* at *penDE* of DS68530	pYN1_78, pLM1_52, pYN1_65, pYN1_79, pICH41780	pAGM4673

Table S5: Primers used for qPCR

Target	Primers
γ-actin	F:TTCTTGGCCTCGAGTCTGGCGG R:GTGATCTCCTTCTGCATACGGTCG
pcbC	F:AGGGTTACCTCGATATCGAGGCG R:GTCGCCGTACGAGATTGGCCG
penDE	F:CATCCTCTGTCAAGGCACTCC R:CCATCTTTCCTCGATCACGC
STF	F:TATATCATGGCCGACAAGCA R:GAACTCCAGCAGGACCATGT

Note S1: Constructed strains and corresponding deposit identifiers

Deposit ID	Parent strain	Transformed DNA and description	Genomic properties	Genes of interests
DS82248	DS68530 (AFF407)	pVE2_1 1xQUAS,pcbC	ΔPenicillin-BGC, Δ*hdfA*, *amdS* marker at penicillin loci	STF (QFDBD-VP16-GFP-NLS), DsRed-SKL
DS82249		pVE2_2 5xQUAS,pcbC		
DS82250		pVE2_3 11xQUAS,pcbC		
DS82255		pVE2_8 pcbC control		
DS82266		pLM2_45 pE, pcbC control		
DS82259		pVE2_12 5xQUAS control		
DS82256		pVE2_9 pcbAB control		
DS82257		pVE2_10 penDE control		
DS82258		pVE2_11 phl control		
DS82265		pLM2_32 NirA control		
DS82264		pLM2_31 P40S-Ura3 control		
DS82252		pVE2_5 5xQUAS,pcbAB		
DS82253		pVE2_6 5xQUAS,penDE		
DS82254		pVE2_7 5xQUAS,phl		
DS82263		pLM2_29_5xQUAS,NirA		
DS82262		pLM2_28_5xQUAS,Ura3		
DS82251		pVE2_4 5xQUAS-reverse, pcbC		
DS82260		pVE2_19 pcbC whole lenght		
DS82261		pVE2_20 pcbAB whole lenght		
DS82244	DS54468 (AFF401)	YN2_71+YN1_81	ΔPenicillin-BGC, Δ*hdfB*, *amdS* marker at penicillin loci	STF (QFDBD-VP16-GFP-NLS), synthetic promoters upstream the penicillin BGC genes
DS82245		YN2_71+YN1_82		
DS82246		YN2_71+YN1_77		
DS82247		YN2_71+YN1_80		

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CHAPTER 3

CRISPR-mediated, vector-based, marker-free genome editing tool for filamentous fungi

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Abstract

Filamentous fungi are highly productive cell factories, often used industrially for the production of a wide range of enzymes and natural products. Genome editing can further help to unlock the potential of transcriptionally silent genes or entire biosynthetic gene clusters, creating even more efficient cell factories for primary- or secondary metabolite production. The limited number of fungal selection markers calls for highly efficient, precise, marker-free genome editing, which is achievable through the CRISPR/Cas9 technology. Here, we present the development of a non-integrative, fungal, CRISPR-based genome editing vector. Several established fungal promoters were evaluated for the successful delivery of the CRISPR components *in vivo* in *Penicillium rubens*. The system expresses both the Cas9 protein from *Streptococcus pyogenes* and the CRISPR single-guide-RNA from an episomal AMA1-plasmid. The created genome editing vector can be used for rapid construction of CRISPR libraries through Golden Gate cloning of various sgRNA target sequences. Genome editing enabled by the constructed CRISPR vector was validated using fluorescent reporters, and as a further proof of concept, on genomic deletion of a recently identified pseudo condensation domain of the L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine synthetase (ACVS) that mediated the formation of the tripeptide precursor for β-lactam antibiotics production.

Keywords: CRISPR, Cas9, marker-free, genome editing, *Penicillium rubens*

Introduction

The fungal kingdom includes many species that have the capacity of producing a wide variety of useful proteins and natural products. Fungal expressed endo- or exogenous proteins are applied in various fields of white and red biotechnology.[1] Moreover, fungi are also known prolific producers of secondary metabolites (SMs), compounds which are not primarily involved in normal growth, development, and reproduction of the organism.[2] The increasing number of sequenced fungal genomes revealed, that fungi hold an untapped reservoir of fungal biosynthetic potential of novel secondary metabolites in the form of transcriptionally silent biosynthetic gene clusters (BGCs).[3,4]

The stimuli, which activate transcriptionally silent promoters are often unknown, and when no known compound is connected to a BGC, these clusters are called "cryptic". Numerous tools have been developed for the activation of transcriptionally silent BGCs for filamentous fungi.[5] In the past, changing cultivation conditions or co-cultivation with different organisms has led to the discovery that one strain is capable to produce many compounds (OSMAC), and that these conditions regulate what subset of BGCs are activated.[6] Genome editing approaches can be utilized for cryptic BGC activation through cluster-specific transcription factor overexpression, knock-out or overexpression of global transcriptional regulators, or more directly by promoter exchange of the gene(s) of interest.[5] With the advent of synthetic biology, new tools for gene regulation became available; including synthetic transcription factors (STFs), transcription activator-like effectors (TALEs), and CRISPR-based (Clustered Regularly Interspaced Short Palindromic Repeats) transcriptional regulation (CRISPRa/i) systems. Although these new technologies each could provide novel approaches for transcriptional regulation, highly efficient and precise genome editing remains essential for metabolic engineering or direct transcriptional activation of cryptic genes. As fungal selection markers are scarce in the fungal synthetic biology toolbox, marker-free (MF) genome editing could allow for rapid sequential genome editing in the same strain lineage.

The CRISPR/Cas system, also referred to as key components of an bacterial immune system, can be used as a molecular tool that provides simple but efficient genome editing as well as transcriptional regulation in a wide range of organisms.[7] In this two component system, the Cas endonuclease is guided by a CRISPR RNA (crRNA) to a target specific locus to introduce a DNA cleavage. In CRISPR/Cas9 systems this crRNA is delivered on a single guide RNA (sgRNA), which consists originally of two separate short RNA transcripts: trans-activating CRISPR RNA (tracrRNA) providing stem loops for the endonuclease protein and CRISPR RNA (crRNA) providing the target sequence.[8] In the Cas9-based (CRISPR-associated protein 9) system the genomic locus is targeted by a target-specific ~20 nucleotide crRNA, which on the complementary genomic target sequence (protospacer) must be followed by a protospacer adjacent motif (PAM). This PAM sequence is unique for different Cas proteins and it represents a limit for possible targets, e.g. it is the NGG three-nucleotide sequence (where N is any nucleotide) in the case of the commonly used *Streptococcus pyogenes* Cas9 (*Sp*Cas9).[9] Careful design of the crRNA is essential to avoid off-target CRISPR effects, as the ribonucleoprotein complex is capable to bind to highly similar sequences[10], which represent another limitation to the possible sgRNA targeting sequences.

As the targeting sequence of the sgRNA can be easily changed, it makes the CRISPR/Cas9 system a simple, flexible, sequence-specific, genome editing tool. At the targeted locus the CRISPR-induced double-stranded breaks can be repaired via different repair mechanisms.[11] Double stranded DNA breakage can be repaired through either the homology-directed repair (HDR), or the non-homologous end joining (NHEJ) pathway. NHEJ-mediated DNA repair can introduce small deletions or insertions at the affected sequence, meanwhile through HDR DNA fragments can be inserted at the targeted locus using homologous flanking sequences upstream and downstream the target, commonly via homologous recombination (HR).

Targeted gene manipulation in wild type *Aspergillus* and *Penicillium* species is challenging, due to the relatively low rates of HR and high rate of

random genomic integration of the transformed DNA. Meanwhile yeasts like *Saccharomyces cerevisiae* favors HR to repair double stranded breaks, most fungi prefer the NHEJ pathway.[12] Targeting efficiency of the donor DNA to the desired location is only 0.1 - 5.0 %, depending on the target locus and the organism.[13,14] Numerous techniques were developed to increase targeting efficiency. Using long homologous flanking sequences (1-2 kb) upstream and downstream of the target shown to increase gene targeting efficiency, reaching 22% in NHEJ proficient *Aspergillus fumigatus.*[15] Applying the split-marker method (known as bipartite gene targeting) also increased the gene targeting efficiency to 60-63% in *A. nidulans* and *A. niger.*[16,17] The split-marker method –initially developed for *Saccharomyes cerevisiae*[18]– delivers two DNA fragments, containing parts of a functional selection marker overlapping each other, as well as homologous sequences for genomic integration.

The core components of NHEJ DNA repair pathway in eukaryotes are the Ku70/Ku80 heterodimer protein complex and the DNA Ligase IV (Dnl4/Lig4).[19] Inactivation of either the fungal homologs of the human *ku70/ku80* (*hdfA*/*hdfB* in *P. rubens, kusA*/*kusB* in *A. niger, and nkuA*/*nkuB* in *A. nidulans* respectively) or *Lig4* (*ligD* in *A. oryzae)* genes results in NHEJ deficiency, which highly increases the efficiency of the targeted DNA integration.[13,20–22] Targeting efficiency of delivering marker-based donor DNA increased from 1% to 47% in the *hdfA* inactivated *P. rubens*[13] meanwhile targeting efficiencies reaching 95-100% in NHEJ deficient Aspergillus[20–22] and *Neurospora crassa*[23].

Highly efficient CRISPR-based genome editing tools have been rapidly developed and established for various organisms, like bacteria, yeast, and human cells.[24] Cas9 based genome editing in filamentous fungi has been established amongst others in *A. fumigatus*, *A. oryzae*, *Neurospora crassa*, *Pyricularia oryzae*, *Trichoderma. reesei*, *Ustilago maydis* and *P. rubens.*[25] CRISPR genome editing can be performed by delivering purified CRISPR/Cas9 proteins and *in vitro* synthesized single-stranded guide RNAs (sgRNAs) in a pre-assembled complex as RNPs, or as *in vivo* CRISPR systems, which relies on expressing these two components by the host organism. As the fungal AMA1 sequence from

A. nidulans supports autonomous vector replication in several filamentous fungal species.[26,27] CRISPR elements expressed by the host can be delivered on a single fungal replicating vector[28–31], or as linearized fragments which recombine *in vivo* in the host.[32] Single vector-based CRISPR/Cas9 genome editing approaches have been developed for several filamentous fungal species.[25] The number of copies of AMA1 vectors maintained in one nucleus might vary in different strains[26], and these vectors are easily lost without maintaining selection[27], which allows for recycling of the same vector-based system.

The short, non-translated sgRNAs are successfully transcribed in various filamentous fungi using RNA polymerase III (Pol III) and RNA polymerase II (Pol II) using ribozyme self-cleaving sequences [reviewed in Song *et al*.[25]]. Pol III promoters are often employed for sgRNA transcription, as the transcripts will not have five-prime cap structures or poly-A tails, unlike Pol II promoter transcripts. Co-transformation of the donor DNA and the CRISPR/Cas9 vector using the exogenous SNR52 Pol III promoter from *S. cerevisiae* for sgRNA transcription showed 25-53% targeting efficiency in *A. fumigatus.*[29] In *U. maydis in vivo* sgRNA delivery was carried out using the endogenous Pol III promoter sequence of the homolog of the human U6 small nuclear RNA 1, resulting in 70% knock-out efficiency of the gene of interest.[33] Meanwhile using an endogenous U6 Pol III promoter in *A. oryzae* provided efficiency lower than 20% in generating desired gene mutations.[34] In a recent study endogenous Pol III tRNA promoters were shown to be providing different efficiency in CRISPR applications in *A. niger.*[30] Some of these tRNA promoters were proven to be highly functional, ~42% efficiency of targeted gene replacement was observed in *A. niger* with a functioning NHEJ machinery and an efficiency of >90% gene replacement in a NHEJ deficient (Δ*kusA*) background. The widely used, constitutive Pol II promoter of *gpdA* (from *A. nidulans*) was shown to be functional in numerous *Aspergillus* species to transcribe a long RNA with hammerhead (HH) ribozyme and hepatitis delta virus ribozymes (HDV) sequences to self-cleavage and release functional sgRNAs[28], providing a solution to use Pol II promoters in the host for sgRNA transcription, through a HH-sgRNA-HDV transcript.

We aimed to establish a versatile, fungal CRISPR/Cas9 vector for genome editing, which provides delivery of the Cas9 protein and the sgRNA from a single construct, and mediating rapid CRISPR library construction. On the analogy of such system, the established elements can be adopted from genome editing to other CRISPR-based applications for transcriptional regulation (CRISPRa/i). In this work, we have evaluated the components of the CRISPR/Cas9 system using fluorescent reporters, comparing different promoters to deliver sgRNAs *in vivo* in *P. rubens*. We established a rapid sgRNA target sequence insertion method, which combines a Golden Gate cloning approach with blue/white screening in *Escherichia coli* to provide convenient cloning of new sgRNA targeting sequences into a non-integrative fungal AMA1 vector, and reducing experimental time to create CRISPR/Cas9 vector libraries. As a proof of concept, we used the system for MF genome editing to remove a recently identified pseudo condensation domain of the L-δ-(ɑ-aminoadipoyl)-L-cysteinyl-D-valine synthetase (ACVS) NRPS gene (*pcbAB*, Pc21g21390) in *P. rubens*, which produces the linear tripeptide (LLD)-ACV, the precursor of all β-lactam antibiotics.

Results and discussion

Expression of SpCas9 in *P. rubens*

To validate the expression of the Cas9 protein from *Streptococcus pyogenes* (*Sp*Cas9) in *P. rubens*, the gene was fused with an enhanced green fluorescent protein (eGFP), tagged with the Simian virus 40 nuclear localization sequence (SV40 NLS, PKKKRKV). This protein fusion was created using the Golden Gate cloning-based Modular Cloning (MoClo) system[35] and was delivered using an AMA1-based, fungal shuttle vector (pLM-AMA-002), which was designed for rapid assembly of MoClo-compatible transcription units and extra-chromosomal expression of genes in filamentous fungi (Figure 1a). This fungal shuttle vector carries a phleomycin fungal resistance marker (*bleo*) and a *lacZ* gene, which can be replaced with MoClo elements, using BsaI restriction sites. The created new AMA1 vector, carrying the P*40S*-SpCas9-eGFP-NLS-T*tif35* transcription unit, was transformed into *P. rubens* and protein expression was validated using

fluorescence microscopy after 5 days of cultivation on phleomycin supplemented solid media. Driven by the constitutive promoter of the 40S ribosomal protein S8 from *A. nidulans* (An0465, 40S, RPS8) nuclear localized GFP was detectable in the fungal strain carrying *Sp*Cas9-eGFP-NLS (Figure 1b), showing successful expression of the GFP-tagged *Sp*Cas9 protein in *P. rubens*.

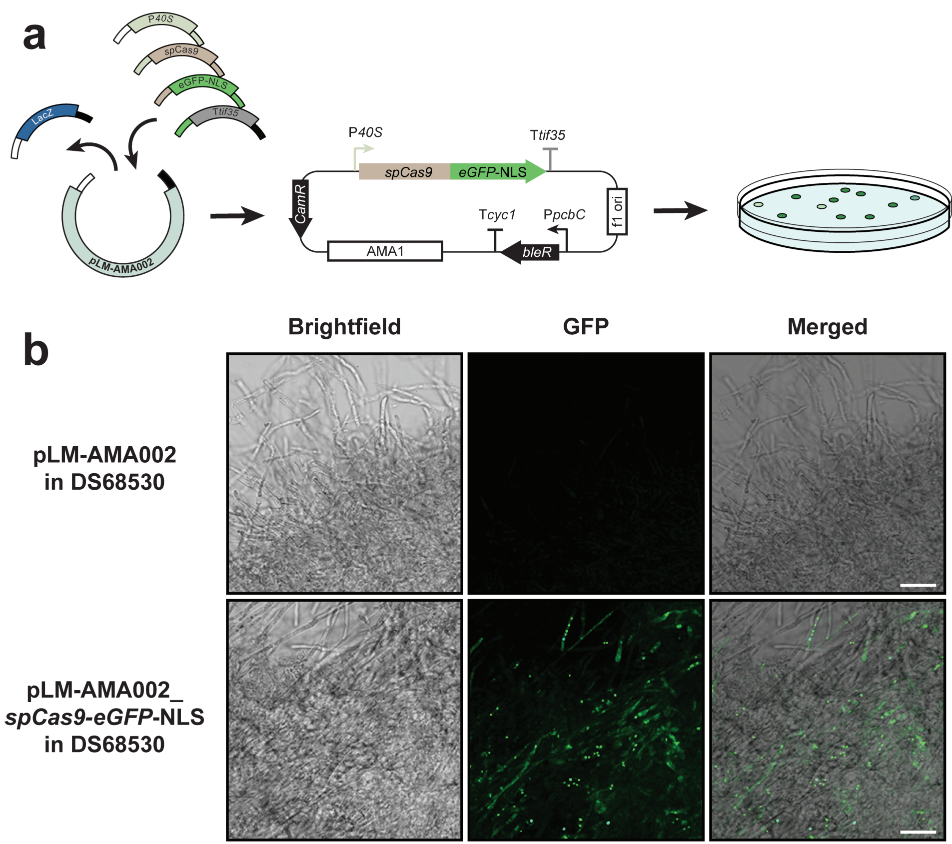

Figure 1. Expression of fluorescently labeled SpCas9 protein in *P. rubens* using the MoClo compatible fungal shuttle vector. a) Schematic representation of restriction/ligation-based Modular Cloning of the *Sp*Cas9-eGFP-NLS expressing transcription unit on pLM-AMA002 shuttle vector. b) Fluorescence microscopy imaging of strains carrying pLM-AMA002 (negative control) and pLM-AMA002_*Sp*Cas9-eGFP-NLS vectors. The fungi were grown for 5 days on phleomycin supplemented solid SMP media. Scale bars represent 20 µm.

Functional sgRNA transcription in *P. rubens*

For the second component of an *in vivo*-expressed CRISPR/Cas9 system in *P. rubens*, promoters for sgRNA transcription had to be identified. One Pol II promoter, P*gpdA* with HH and HDV ribozyme self-cleaving sequences and three Pol III promoters (U6 from *U. maydis*, SNR52 from *S. cerevisiae*, tRNA[Ser] from *P. rubens*) were investigated for sgRNA expression in *P. rubens*. To evaluate functional sgRNA promoters, multigene transcriptional cassettes were constructed to express both *Sp*Cas9 and sgRNA in combination for genome editing. The transcriptional unit cassettes were integrated on the genomic DNA on *P. rubens* using homologous recombination, using flanking sequences homologous to the locus of *PKS17* (Pc21g16000) in *P. rubens* DS82249 strain (Figure 2a). The *P. rubens* DS82249 strain is derived from the industrial DS68530[36] and expresses a rapidly maturing red fluorescent protein (DsRed) with a peroxisomal localization tag (SKL) under the control of a synthetic transcriptional factor[37], QfDBD-VP16AD (5xQUAS-CP*pcbc*-DsRed.T1-T*ACT* cassette integrated at the penicillin BGC locus). In all designed expression cassettes with different promoters for sgRNA expression, the sgRNAs were designed to target the ORF of the *DsRed* gene located in the penicillin locus of DS82249 (Figure 2b) and the MF donor DNA was used for HR-mediated knock-out of the *DsRed* gene (Figure 2c). Functional sgRNA delivery was evaluated based on the percentage of the colonies which lost the function to produce DsRed protein, due to CRISPR-mediated double-stranded DNA breakage, inducing disruption of the DsRed ORF and/or the incorporation of the MF donor DNA via HR.

A loss-of-function to produce DsRed fluorescent protein was detected in up to 13% of the colony forming units after transformation without sgRNA transcription unit (non-sgRNA negative control), similarly to when the sgRNAs were transcribed by the U6 from *U. maydis* and SNR52 from *S. cerevisiae* exogenous Pol III promoters. As the presence of these sgRNA transcription units did not increase the number of *DsRed* knock-outs compared to the non-sgRNA negative control, we consider these sgRNA transcription units non-functional in *P. rubens.* On the other hand, an increased loss of 73 ± 23% and 62 ± 28% in

fluorescent colonies was detected when the sgRNA was transcribed under the control of the native *P. rubens* Pol III tRNA[Ser] promoter or the P*gpdA* Pol II promoter (HH-sgRNA-HDV) (Figure 2d).

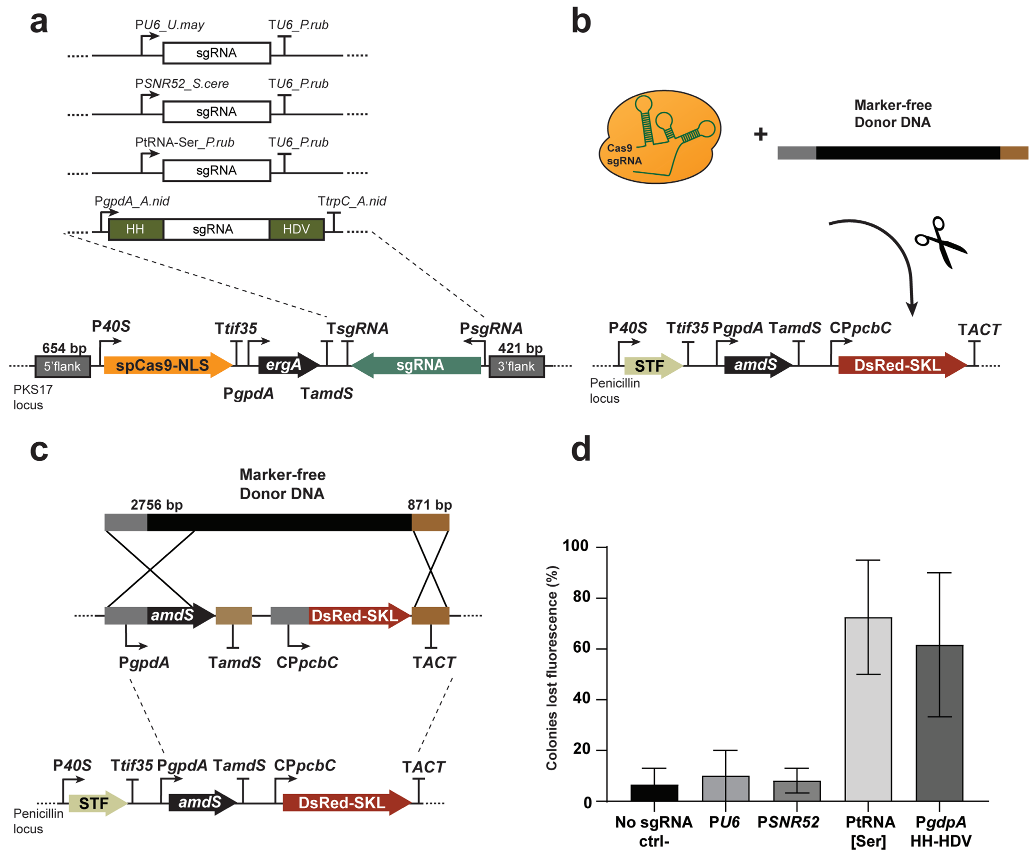

Figure 2. Overview of the promoter evaluation approach for CRISPR-sgRNA expression in *P. rubens*. a) Schematic representation of modular multi-gene DNA constructs carrying terbinafine resistance marker, SpCas9 and various sgRNA transcription units delivered via homologous recombination to the PKS17 locus of *P. rubens* DS82249. b) Representation of *in vivo* expressed CRISPR RNP components targeting the ORF of DsRed-SKL on the penicillin locus. c) Illustrated incorporation of the marker-free donor DNA via homologous recombination and marker-free knock-out of DsRed. d) Percentage of colonies that lost DsRed expression after transformation with a combination of different sgRNA transcription units and SpCas9. Data shown are the means 2 separate experiments examining 30-40 randomly selected colonies, vertical bar represents ± standard error.

The observed high knock-out efficiency with sgRNA expression under the control of the native tRNA[Ser] Pol III promoter from *P. rubens* is in line with the results in *A. niger*, where from a selection of 37 endogenous tRNA promoters, 36 shown to be functional for sgRNA delivery[30] while the Pol II *gpdA* promoter in combination with the HH-sgRNA-HDV transcription system has been shown to be functional in various fungal species[25,38]. For further experiments, *gpdA* promoter was used for sgRNA transcription in combination with HH and HDV rybozymes, aiming at maximal compatibility with other fungal strains.

Single vector-based sgRNA and SpCas9 expression and genome editing in *P. rubens*

A CRISPR-based genome-editing AMA1 vector was constructed (pLM-AMA15.0), carrying the previously evaluated *Sp*Cas9 and sgRNA expression units on a single fungal replicating vector. This single vector contains the two components of the CRISPR/Cas9 system and also carries phleomycin (*bleR*) and terbinafine (*ergA*) fungal selection markers. To enable convenient and efficient exchange of sgRNA target sequences, a sgRNA "plug-and-play" module (P*gpdA*-*lacZ*-HDV-T*trpC*) was introduced into the AMA1 shuttle vector to facilitate rapid cloning in *E. coli* (Figure 3). Using this method, sgRNA transcription units can be assembled on a fungal shuttle the vector and positive bacterial clones can easily be detected with blue-white screening. The sgRNA backbone sequence and the HDV ribozyme are already integrated on the AMA1-vector together with a *lacZ* gene flanked by BsaI restriction sites. The 20 bp spacer sequence defining the genomic target is supplied on a separate double-stranded DNA molecule, together with the HH ribozyme which includes the necessary 6 bp inverted repeat of the 5'-end of the spacer to complete the HH cleavage site. The DNA fragment can then be inserted into pLM-AMA15.0 using the Golden Gate cloning and the BsaI restriction sites (Figure 3a).

To evaluate the created AMA1-based CRISPR vector for genome editing, CRISPR sgRNA spacer insert was designed to target the ORF of the DsRed (Figure 3a) in *P. rubens* DS82249. The vector containing both components of

the CRISPR/Cas9 system was transformed without any additional donor DNA, and successful genome editing was evaluated based on the percentage of DsRed negative colonies (Figure 3b). None of the 60 analyzed DS82249 transformants carrying the pLM-AMA-15.0 (no sgRNA control) lost DsRed fluorescence. After transformation of DS82249 with pLM-AMA-15.DsRed (sgRNA targeting the DsRed OFR), 7 out of the total 60 colonies that lost their capacity to produce DsRed. To

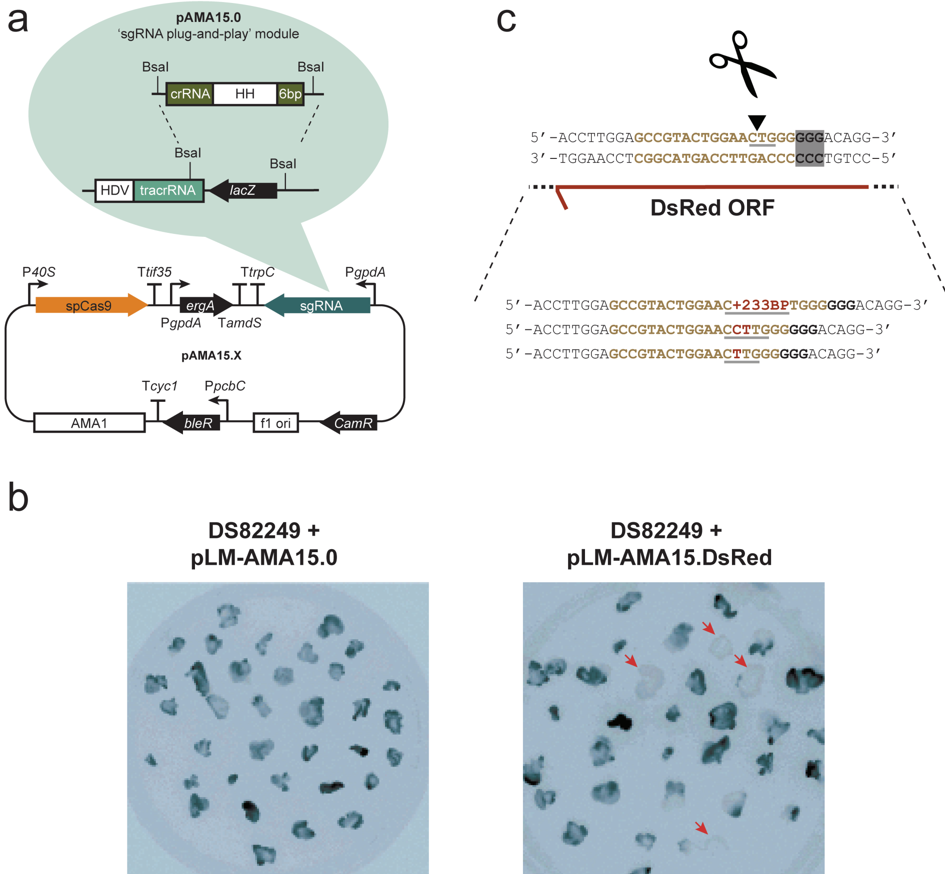

Figure 3. CRISPR/Cas9 vector-mediated genome editing without additional donor DNA in DsRed-SKL fluorescent reporter expressing strain. a) Representation of pLM-AMA15.0 CRISPR vector, highlighting the *gpdA* promoter-driven sgRNA "plug-and-play" module. b) Fluorescent imaging of *P. rubens* DS82249 strain, maintaining pLM-AMA15.0 (non-sgRNA negative control) and the DsRed ORF targeting AMA15.DsRed vectors. Red arrows indicate colonies without red fluorescence. c) Visual representation *in vivo* expressed CRISPR components targeting the DsRed ORF, initiating double-stranded DNA breakage upstream the PAM sequence. Mutations revealed by sequencing on the DsRed ORF in colonies without DsRed expression.

evaluate the targeting efficiency of the sgRNA-Cas9 complex, the DsRed ORF was sequenced. Sequencing results showed different insertions (T, CT nucleotides, or in one case a 233 bp sequence from the Pc21g20360 gene) in the ORF of DsRed, 3 bp upstream the PAM sequence (Figure 3c). These results confirmed that the genome editing event was CRISPR/Cas9-mediated, and the double-stranded DNA breakage was most likely repaired through NHEJ DNA repair mechanism.
Our detected insertions in the DsRed ORF in a *hdfA*-deficient *P. rubens* point towards an *hdfA*-independent NHEJ DNA repair mechanism. An alternative Ku-complex-independent but Lig4-dependent NHEJ pathway was suggested to be present in *P. rubens*[39], *N. crassa*[23] and *M. grisea*[40].

Comparison of CRISPR vector and RNP-based marker-free genome editing methods in *P. rubens*

To validate the applicability of the AMA1 vector-based system CRISPR-mediated MF genome editing was carried out on the *pcbAB* gene (ACVS) of the penicillin gene cluster of *P. rubens* DS54468 (1×penicillin-cluster, Δ*hdfA*, derived from DS47274)[36] (Figure 4). The results were compared to the previously established *in vitro* synthesized ribonucleoprotein (RNP)-based MF genome editing.[32] The MF donor DNA was designed to remove an unidentified pseudo-condensation domain at the N-terminus of *pcbAB* by HDR, using 106 bp homologous flanking sequences upstream and downstream the target for HR (Figure 4b). From each transformation plates 16 colonies were tested for the correct integration of the MF donor DNA. Removal of the predicted pseudo condensation domain of ACVS (*pcbAB*) was confirmed by colony PCR and DNA sequencing. Whereas the AMA1-vector-based, *in vivo* expressed genome editing CRISPR system produced 2/16 correct clones, the *in vitro* synthesized RNP editing method produced 7 correct clones out of 16 selected transformants (Figure 4c). Colonies with correct integration lost their ability for ACV formation. The effects of the removed pseudo condensation domain of ACVS on penicillin production are further discussed in [Iacovelli *et al*.[41]].

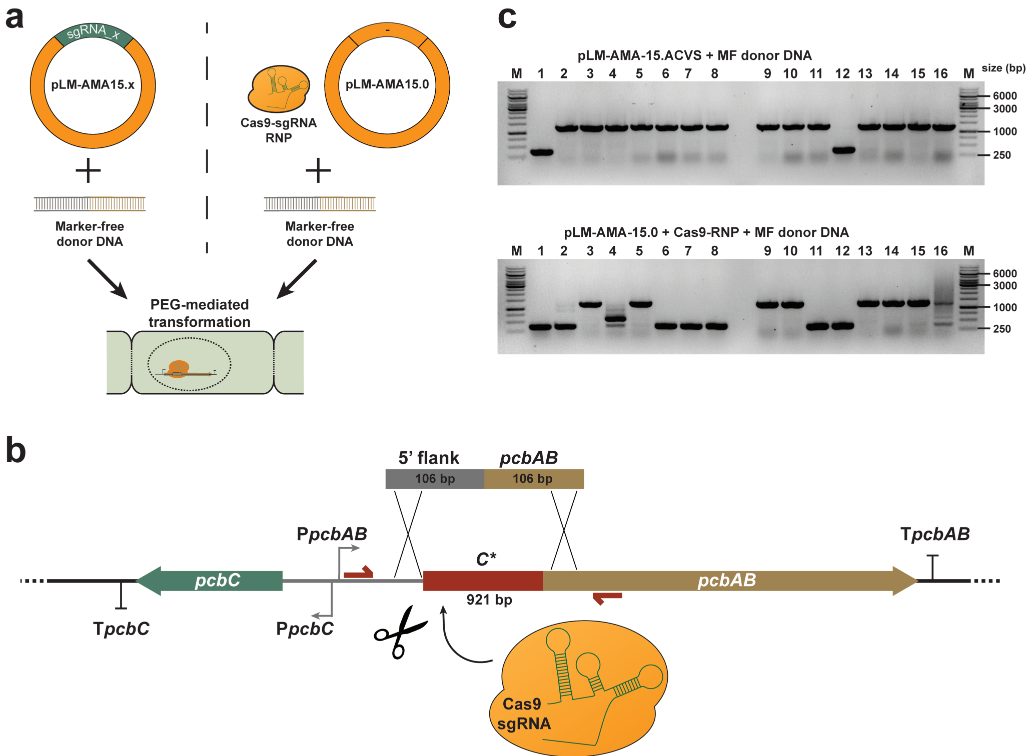

Figure 4. CRISPR/Cas9-mediated, marker-free genome editing. a) Representation of the delivery of sgRNA expressing CRISPR/Cas9 vector, and *in vitro* synthesized RNP components for marker-free, CRISPR-mediated genome editing in *P. rubens*. Fungal selection marker (*ergA*) is provided on the non-integrative AMA1 vector. b) Illustration of CRISPR/Cas9-based marker-free genome editing and removal of pseudo-condensation domain from *pcbAB* of *P. rubens* DS54468. Red arrows indicate primer binding sites for PCR, outside the pseudo condensation domain. c) PCR analysis on transformants engineered using CRISPR/Cas9 vector pLM-AMA15. ACVS (targeting *pcbAB*) (upper lane) and RNP-based CRISPR/Cas9 method (bottom lane). When the pseudo-condensation domain (C*) sequence (1251 bp) is replaced with the marker-free donor DNA, a short PCR fragment (330 bp) is expected.

Both the vector-based delivery and the *in vitro* synthesized RNP-based delivery of the CRISPR components resulted in correctly integrated MF donor DNA, indicating that the CRISPR system was able to mediate targeted gene integration with short homologous flanking sequences (106 bp). The difference in targeting efficiency of the two method might be due to the amount of CRISPR components present at the moment of transformation, and using either of the

systems can be more advantageous depending on the purpose of the experiment. Synthetic RNPs with Cas9 protein are preferred for genetic modifications, where the presence of CRISPR components is not needed after the genome editing event. Eventually, the RNP components will be lost after transformation when the cells propagate. This method, however, requires access to the purified Cas9 protein. The *in vivo* delivery of CRISPR components can provide an alternative to the time-consuming preparation or purchasing of *in vitro* synthesized RNP components. Vector-based alternatives provide delivery over a longer time or at different time points (in combination with inducible promoters) when desired. Although advanced sgRNA target sequence prediction algorithms evaluate potential on- and off-target binding effects, predicted and observed efficiencies may vary, making library construction and experimental testing necessary.

Many factors can influence the CRISPR/Cas9 gene editing efficiency, such as the expression efficiency of sgRNAs, codon usage and expression strength of the Cas9 endonuclease, on and off-target binding of the crRNA, or accessibility on the targeted locus. Varying targeting efficiencies from low to high were observed for 6 different *kusA+ Aspergillus* species using CRISPR/Cas9 vector pFC331-pFC334 without additional donor-DNA for HR.[28] These varying results were attributed to the use of an *A. niger* codon-optimized *SpCas9* sequence, and differences in the AMA1 propagation capacity and number of nuclei in the different hosts. Expression of functional sgRNAs from pFC332 resulted in massive cell death in NHEJ deficient *A. niger, A. nidulans and A. oryzae* strains, and from this lethality the transformants were rescued by providing a donor DNA that serves as a repair template in HR repair of Cas9-induced double stranded breaks (also called rescue cassette).[42] This lethality in these strains was advantageous to reduce false positive colony forming units.

In *P. subrubescens*, the MF deletion of the homolog of the *ku70* gene using the pFC332 CRISPR/Cas9 vector with 1kb-long flanking sequences resulted in a low knockout efficiency (2 out of 22 transformants).[31] Recently, pLM-AMA15 CRISPR vector was applied for genome editing in the non-model *P. digitatum* and *P. expansum* strains with 10.1% and 12.7% targeting efficiency, respectively.[43]

Next to genome editing, the identified components of this vector system should be suitable for CRISPRa and CRISPRi applications, by designing transcriptional activation and repression systems in filamentous fungi, respectively. Direct fusion of the catalytically inactive Cas9 (dCas9) protein with a transcriptional regulator would create a synthetic regulator, which can be guided by sgRNAs to the desired gene for transcriptional modification. Using modular cloning systems, such protein fusions can be rapidly assembled e.g. dCas9 with VP16, VP64 or VPR for transcriptional activation[44–46], P300 for histone acetylation-based epigenome editing[47,48], or with Mxi1 and Krüppel-associated box (KRAB) for transcriptional repression[49].

Conclusions

Here we report on a vector-based CRISPR/Cas9 system for filamentous fungi, which enables rapid sgRNA library assembly and efficient, precise genome editing. The vector system provides an alternative to the purchasing or preparation of synthesized CRISPR components. We used established fungal promoters and terminators to express the elements of the system to increase portability to other fungal species. The CRISPR/Cas9 vector was applied for HR-based MF genome editing by removing an N-terminal pseudo condensation domain of the *pcbAB* gene (Pc21g21390, ACV synthetase) using short flanking regions (106 bp) for HR.

With this work, we further expand currently available tools for genome editing in *P. rubens*. As the vector backbone supports autonomous replication in several filamentous fungal species, and as we use established genetic elements to deliver CRISPR components, we expect the vector to be transferable to other fungal species. The pLM-AMA15 vector offers new opportunities for genome modification, it supports the recycling of the vector and provides a consistent CRISPR activity over longer period of time. Steady expression of CRISPR components is essential for CRISPR-based transcriptional regulation systems, such as CRISPRi and CRISPRa, from which the later could be used as a tool for transcriptional activation of cryptic BGCs.

Materials and Methods

Chemicals, reagents and oligodeoxyribonucleotides

All medium components and chemicals were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands) or Merck (Darmstadt, Germany). Oligodeoxyribonucleotide primers were obtained from Merck. Enzymes were obtained from Thermo Fisher Scientific (Waltham, MA) unless otherwise stated. For the design of expression casettes, *in-silico* restriction cloning, and inspection of Sanger sequencing results, SnapGene (GSL Biotech) was used.

Fungal strains and transformation

Penicillium rubens DS68530 and DS54468 strains were kindly provided by., DSM Sinochem Pharmaceuticals (now Centrient Pharmaceuticals B.V), the Netherlands (See Table 1). Protoplasts of *P. rubens* were obtained 48 h post spore seeding in YGG medium and transformed using methods and media described previously.[32]

Strain	Genotype	Derived from	References
DS68530	ΔPenicillin-cluster, Δ*hdfA*	DS17690	[36]
DS82249	ΔPenicillin-cluster, Δ*hdfA, amdS*+ STF-driven *DsRed-T1.SKL expression* (LM2_30)	DS68530	[37]
DS54468	1× Penicillin-cluster, Δ*hdfA*	DS47274	[36,50]

Table 1: Fungal strains used in this study with their corresponding genotypes**.**

Preparation of *in vitro* synthesized CRISPR/Cas9 ribonucleoproteins (RNPs) for PEG-mediated CRISPR/Cas9 genome editing was performed as described previously.[32] Overlapping oligonucleotide sequences used to create templates for T7-based *in vitro* sgRNA transcription for genome editing can be found in Supp. Table S3. Fungal and bacterial strains, media composition, protoplast generation, and PEG-mediated fungal transformation using either phleomycin or terbinafine markers was carried out as described previously.[46] A list of fungal strains created in this study with corresponding transformed donor DNA can be found in Supp. Table S1.

Vector construction

The Golden Gate technology-based Modular Cloning (MoClo) system[35] using Type IIS BpiI and BsaI restriction enzymes was employed for the construction of all vectors unless stated otherwise. PCR amplifications were conducted using KAPA HiFi HotStart ReadyMix (Roche Diagnostic, Switzerland) according to the instructions of the manufacturer. Synthetic or PCR amplified DNA fragments can be found in Table S2. Constructed vectors with their destination vectors with corresponding cloned DNA donor vectors or DNA fragments can be found in Supp. Table S3. Internal BsaI, BpiI recognition sites were removed for MoClo compatibility. Synthetic DNA parts were ordered from IDT.

Cas9 protein expression was evaluated using a GFP reporter to visualise nuclear expression in *P. rubens*, facilitated by the NLS tag on the C-terminal. Cas9-NLS was amplified from synthetic SpCas9 pYTK036 template, provided as part of the Yeast MoClo Toolkit (AddGene ID #65143)[51] to exclude potential incompatibility using human codon-optimized sequences.[52] Cas9 expression was placed under the control of the constitutive 40S promoter (AN0465, 40S ribosomal protein S8 promoter), amplified from pVE2_10_P40S_QfDBD_VP16AD (AddGene ID #154228)[37]. The sgRNA "plug-and-play" transcription unit was constructed as described previously[46]; using HH and HDV ribozyme sequences (self-cleavage), *gpdA* (AN8041) promoter and *trpC* terminator sequences amplified from pFC334 (AddGene ID # 87846)[28]. The terbinafine resistance gene (*ergA*) was amplified from *P. rubens* DS6853 genomic DNA (gDNA)[53].

For sgRNA promoter evaluation, we have delivered the two components of the *in vivo* CRISPR/Cas9 system (Cas9 and sgRNA) using a terbinafine marker to the PKS17 locus of a DsRed producing DS82249 *P. rubens* strain[37] using PEG-mediated transformation and HR. The MF donor DNA (BD1_10) was designed to target the DsRed transcription unit using homologous flanking regions upstream and downstream (2.7 and 0.8 kbp respectively) for HR. During the PEG-mediated co-transformation the MF donor DNA was supplemented in great excess (100:1 molar ratio) compared to the terbinafine marker-based DNA fragment carrying the CRISPR components in the total amount of 10 µg transformed DNA.

The pLM-AMA002 autonomously replicating shuttle vector, carrying the AMA1 sequence, was constructed on the backbone of the pDSM-JAK-109 vector[54] where the P*gpda*-*DsRed*-SKL-T*penDE* transcriptional unit was removed using the BspTI and NotI restriction enzymes.[46] The linear vector was treated with the Klenow Fragment and ligated to the circular vector using the T4 DNA Ligase according to the instructions of the manufacturer, creating a new AMA1 vector (pLM-AMA001) carrying a phleomycin resistance marker, but no *DsRed* expression cassette. This vector was linearized and cloned with a removable *lacZ* gene cloning site using BspTI, based on the "level 1 transcription unit" receiver backbones of the MoClo system, creating pLM-AMA002. This receiver vector was brought into a Golden Gate Assembly together with MoClo compatible entry vectors, to assemble the transcription unit of P*40S*-*SpCas9-NLS*-T*tif35* on pLM-AMA002 (Supp. Table S3).

The pLM-AMA15.0 CRISPR/Cas9 vector with sgRNA expression unit was constructed using Modular Cloning[35] and Gibson Assembly[55]. The pLM-AMA001 vector was linearized using BspTI and was brought into Gibson Assembly together with the terbinafine marker, *SpCas9-NLS* expression unit and the previously established P*gpdA*-HH-sgRNA-HDV-T*TrpC* "plug-and-play" transcription units, creating pLM-AMA15.0 as described previously[46]. Full sequence of pLM-AMA15.0 is available at the AddGene repository under AddGene ID #138944.

Fluorescence imaging

Fungal strains carrying the SpCas9-eGPF-NLS expression vector were further cultivated after transformation on phleomycin-supplemented (50 µg/ml) transformation solid medium for 5 days and examined using fluorescence microscopy. A small amount of hyphae was taken from the peripheral zone of the colonies and suspended in phosphate-buffered saline (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, pH 7.3). The nuclear localised GFP signal was visualized by excitation with a 488 nm argon laser (Lasos Lasertechnik, Jena, Germany), and emission was detected using a 509 nm bandpass emission filter. Fungal strains carrying the DsRed.SKL expression cassette were further cultivated after

transformation on terbinafine-supplemented (1.1 μg/ml) transformation solid medium for 5 days and the colonies were examined for fluorescence protein expression using Molecular Imager ChemiDoc XRS.

Abbreviations

SM, secondary metabolite; BGC, biosynthetic gene cluster; MF, Marker-free; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas9, CRISPR associated protein 9; sgRNA, single guide RNA; crRNA, CRISPR RNA; PAM, protospacer adjacent motif; eGFP, enhanced green fluorescent protein; NLS, nuclear localization sequence; DsRed.T1, red fluorescent protein T1 variant; SKL, serine-lysine-leucine peroxisomal targeting signal

Author contributions

L.M. designed and carried out all experiments and wrote the manuscript with critical feedback and help from R.A.L.B., Y.N. and A.J.M.D.

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Supplementary information

Transformed strain	Transformed donor DNA	Identifier	Purpose
DS68530	pLM-AMA002	LM-AMA002	Empty vector control for pLM-AMA002
DS68530	pLM-AMA002_P40S-spCas9-eGFP-NLS-Ttif35	LM-AMA002_P40S-*sp*Cas9-eGFP-NLS-Ttif35	spCas9-eGFP expression cassette on pLM-AMA002
DS82249	pLM2_100 and Part ID "34"	gpdA-No_sgRNA_negCTRL	Non-sgRNA / negative control for sgRNA promoter evaluation
DS82249	pLM2_101 and Part ID "34"	U6_U_may	P*U6* (from *U. maydis*) for sgRNA (Pol III) promoter evaluation
DS82249	pLM2_102 and Part ID "34"	SNR52_S_cere	P*SNR52* (from *S. cerevisiae*) for sgRNA (Pol III) promoter evaluation
DS82249	pLM2_104 and Part ID "34"	tRNA_P_rub	PtRNA[Ser] (from *P. rubens*) for sgRNA (Pol III) promoter evaluation
DS82249	pLM2_127 and Part ID "34"	gpdA_A.nid	P*gpdA*+HH+HVD (*A. nidulans*) for sgRNA (Pol II) promoter evaluation
DS82249	pLM-AMA15.0	LM-AMA15.0	Non-sgRNA / negative control for single CRISPR/Cas9 vector-based KO evaluation (DsRed)
DS82249	pLM-AMA15_DsRed_KO	LM-AMA15_DsRed_KO	Fully assembled CRISPR/Cas9 vector, sgRNA targeting DsRed ORF
DS54468	pLM-AMA15.0 and Part ID "35"	LM-AMA15.0+RNP ACVS	Non-sgRNA / negative control for single CRISPR/Cas9 vector-based KO evaluation (ACVS)
DS54468	pLM-AMA15_ACVS_KO and Part ID "35"	LM-AMA15.ACVS_KO	Fully assembled CRISPR/Cas9 vector, sgRNA targeting ACVS ORF

Table S1. List of fungal transformations performed in this study with their corresponding transformed DNA

Seq. ID	Description	Sequence (5′ to 3′)	Template
1	pZB0_21 p40S A	F:TTGAAGACAAGGAGGAGTTATAGACGGTCCGGCATAGG R:TTGAAGACAAGGTGTCGATCGGACGTATTGTCCAAG	pDSM-JAK108
2	pZB0_21 p40S B	F:TTGAAGACAACACCAAAGCAATCTGGTACATCACCC R:TTGAAGACAAGGTATCCTCCACAGACTCCTTGAGCC	pDSM-JAK108
3	pZB0_21 p40S C	F:TTGAAGACAATACCTGATATAAGAATTGAGAGTTATACTCCGG R:TTGAAGACAACATTGTTTGCTGTCTATGTGGGGGACTG	pDSM-JAK108
4	pLM0_23 spCas9 direct fusion	F:TGAAGACTTAATGGACAAGAAGTATTCTATCGGACTGG R:TGAAGACTTACCTATCCCCTCCGAGCTGTGAGAG	pYTK036 (AddGene #65143)
5	pDL0_9 eGFP-NLS direct fusion	F:AGAAGACAAAGGTCCGTGAGCAAGGGCGAGGAG R:TGAAGACAATACCTTAGACCTTCCGCTTCTTCTTTGGCTTGTA	pLM2_30 (AddGene #154222)
6	pLM0_40 spCas9-NLS Stop codon	F:TGAAGACTTAATGGACAAGAAGTATTCTATCGGACTGG R:TGAAGACTTAAGCTTATACCTTTCTCTTCTTTTTTGGATCTACCTTTCT	pYTK036 (AddGene #65143)
7	pYN0_10 tTif	F:ACAGCGGAAGACAAGCTTACTTCTTTATCGGTTCTCTCTTACGAC R:ACAGCGGAAGACAAAGCGGTGCTTGGGATGTTCCATGGTAGCTGTG	pDSM-JAK108
8	pLM0_12 P*gpda*	F:ACAGCGGAAGACAAGGAGTGGATCCCCCGGGCTGCAGG R:GAAGACTTCATTGTGATGTCTGCTCAAGCGGG	pDONR221-AMDS
9	pCP0_30 ergA A	F:GGAAGACAAAATGATGACCTTGCTCAATGGCCACG R:GGAAGACAATTCATGGGTGCCGATCTGGTAC	gDNA *P. rubens* DS54468
10	pCP0_30 ergA B	F:GGAAGACAATGAAACCCGAATCCTTATCGATATCCCTG R:GGAAGACAAAAGCTCAGTACCAGATCTCGGTCCAGATG	gDNA *P. rubens* DS54468
11	pLM0_11 amdS A	F:GAAGACTTAATGCCTCAATCCTGGGAAGAAC R:GAAGACAAAAGCCTATGGAGTCACCACATTTCC	pDONR221-AMDS
12	pLM0_11 amdS B	F:GAAGACTTAATGCCTCAATCCTGGGAAGAAC R:TGAAGACAAGTCATCCGCAGGCAGCGTCTG	pDONR221-AMDS
13	pLM0_11 amdS C	F:TGAAGACAATGACAGCGTTATTGATTTCCCAAAGAAATCG R:TGAAGACAAGGTGTCTTGTGCTTTGCGTAGTATTCA	pDONR221-AMDS
14	pLM0_11 amdS D	F:TGAAGACAACACCCGTTGGTCCACTCCAT R:TGAAGACAAGAAAACGGCACCGGCTTTGCGG	pDONR221-AMDS
15	pLM0_11 amdS E	F:TGAAGACAATTTCTACGTCAAGACCTCTGTCCCG R:ACAGCGGAAGACAACATTACTTCATCAGTGACTGCCCGTCTCGTATATAG TATAAAA	pDONR221-AMDS
16	pLM0_11 amdS F	F:TTGAAGACAAAATGTTAGACCTCCGCCTCTTCAC R:TTGAAGACAAGGTATGACCGGCTTTGGCGAGTGCG	pDONR221-AMDS
17	pZB0_20 TamdS	F:ACAGCGGAAGACAAGCTTCTAATAAGTGTCAGATAGCAAT R:ACAGCGGAAGACAAAGCGTACCGCTCGTACCATGGGTT	pDONR221-AMDS
18	pYN0_9 Tact	F:ACAGCGGAAGACAAGCTTGTGCTTCTAAGGTATGAGTCGCA R:ACAGCGGAAGACAAAGCGCGCAGGGTTTGAGAACTCCGATCT	pDSM-JAK109
19	pLM1_87 PKS17 5′flank	F:TGGTCTCTGGAGTCTCTTGTCTATGATCCCACTATCTCATGAC R:TGGTCTCTAGCGAGTCTCCCCAGTGGCGAATTA	gDNA *P. rubens* DS54468
20	pLM1_88 PKS17 3′flank	F:AGGTCTCTGGAGAAGAGCATTGCATTTTGGGGC R:TGGTCTCTAGCGTCTCGGATGCAACGGTATCTGAG	gDNA *P. rubens* DS54468

Seq. ID	Description	Sequence (5′ to 3′)	Template
21	tracrRNA tail	CGGTCTCAGTTTCAGAGCTAGGCCAACATGAGGATCACCCATGTCT GCAGGGCCTAGCAAGTTAAAATAAGACTAGTTCGTTATCAACTTGG CCAACATGAGGATCACCCATGTCTGCAGGGCCAAGTGGCACCGAG TCGGTGCTTTTTGCTTGCTCAGTTATAGTTCTAGGGTACGAGCTTTTT GTGTATGTTGTATATCATTGCGATGTTATACTGTGGATGTGGATATCAC GTGTCCATCGCCGTTCGCTCTCCATGTTGTCACGGTATATTTTGGAAA CTTTAAGGATCAGTCCTTCAGGGCATACCGTGAAGTGAAAATATGTAG AGAGGATTAGCAAGGCCCCCTACTATTCTTCTCCTTCGACATCCTTTCC TAAGGTGTTTATTTCTTGTTATTAATAAACTTGCTTTTTATATTTGTTGGTA TTCTATTATGTATCCAGCTCTCGGCAATTTTATATAAAGTCCAATGAAAAG AAAATATCGTAGAACTCGTCAATTGCATTAATGATGCCCTCACAAGAGGA ACGTAATAGCTGCAGACATCTCCTGAGACCG	synthetic
22	pLM1_91 A	CGGTCTCTAGCGTAATACGTTCGTTCCGATGTAGCTCAGGAACGGGCGC AGAGGCGGATGAATTGGTTGCGGTGGCTATGGATGGTGGAGGTGAACG GCGACGTGCAGCGTCGGTCACAGAGGATCGGATGGGGCCTGTCGGGAT GACCTGTACTATAACGAGGGAGGAGGGGGAGGGAGGAGGGGAAAGAG GAATGTGGGGAAGGCACGTTACCAAGGTTTTGCCAAGAGGCTCTTGTGT TATTCCGGAGGTGTAGCCGAATGGGCGATCCGAGCTAACGCCAGCTGGG CGTGAGAAGCAGTTGGTCTGAAAGCGGAGGCGGTGCAAGACGGTTCTA AGAAGGAGGCGAATAAGAAGTGTTTTGTGTGCTGCGGGTGCGAATAGAC GGTCACGAGTGGATGGAAGCCGACTTGTAGGCGTGCTGAAAGACGTCG TGCGGGTGCGGTTTTGGTTTTGTGTTGGTCTTGGTAAAAGTGTGCCGCA GGTGAGGGTTCTTGATTGGTGAACGTGAAAACGGATGGCCAAGTCCGA GTCGACCAGAGAGAGAGGCAGAGAGAGAGAGAGAGATGGCTCTACAG CGCCGCTTCGCGCGTCCTTCACGATTATTCGTGATTACTGTCCACCGGT CCCTCTTACTCAGAACTGCCGGAACGAATTCGTGATTTACACCAAACAC GCGCTGTCACAGTCACGAGTCCATGAGCCGTGAGCCCCGCTTCAGATC CTGTTTTCTCTTATTCAGCGTAACAACACAAAACAGAATTTCTTCTAAACA CCCTTGCAATTCGCGCACACCCCTGTAGCAGTCTGTCAGCATTCAAAATT CCATTCTACAACGCCCAGTTCCAGTACGGCTCCGTTTTGAGACCGR	synthetic
23	pLM1_92 A	CGGTCTCTAGCGTCTTTGAAAAGATAATGTATGATTATGCTTTCACTCATA TTTATACAGAAACTTGATGTTTTCTTTCGAGTATATACAAGGTGATTACAT GTACGTTTGAAGTACAACTCTAGATTTTGTAGTGCCCTCTTGGGCTAGC GGTAAAGGTGCGCATTTTTTCACACCCTACAATGTTCTGTTCAAAAGAT TTTGGTCAAACGCTGTAGAAGTGAAAGTTGGTGCGCATGTTTCGGCG TTCGAAACTTCTCCGCAGTGAAAGATAAATGATCGCCCAGTTCCAGT ACGGCTCCGTTTTGAGACCGR	synthetic
24	pCP1_82 A	CGGTCTCTAGCGATATTACTACCAAGTAACTAATTTACCACGGAATATAAC GCAAATCACCACATGTCTTGAAATCCCACAATGGACATAACTATTTAAGG CTTTCCTTCACAGGCCTTAGAGAGCTTGGAGAATGCAAAGGCTTCGTAA TCTAGGGAAATAGAGGGCTTATTCGCAATAGACCAAACTTCCCATTGCC TCCCTAGTATAGTATTGGGGAAAAAAAAAAAATCCGGACGTACTTAAGA AGAACATTGAATACGGCGGTTTGGCCGAGTGGTCTAAGGCGATTGAC TCGAACAGAGCATGCAAATTTCCGTTCTCCTTCTCAAGGGAATGAAAT CATATGTTGTATGTGTTCTTGAAATCAATTCCGTTCGCGGGCGCATGTT CGAATCATGCAGCCGTAGAGACCG	synthetic
25	pCP1_82 B	CGGTCTCTCCGTCGTGGAGCCGTACTGGAACTGGGGTTTCAGAGCTAG GCCAACATGAGGATCACCCATGTCTGCAGGGCCTAGCAAGTTAAAATAA GACTAGTTCGTTATCAACTTGGCCAACATGAGGATCACCCATGTCTGCA GGGCCAAGTGGCACCGAGTCGGTGCTTTTTGCTTGCTCAGTTATAGTT CTAGGGTACGAGCTTTTTGTGTATGTTGTATATCATTGCGATGTTATACT GTGGATGTGGATATCACGTGTCCATCGCCGTTCGCTCTCCATGTTGTC ACGGTATATTTTGGAAACTTTAAGGATCAGTCCTTCAGGGCATACCGT GAAGTGAAAATATGTAGAGAGGATTAGCAAGGCCCCCTACTACTCC TGAGACCG	synthetic
26	pLM1_95 A	F:CGGTCTCTAGCGGCGTAAGCTCCCTAATTGGCCC R:CGGTCTCTAAACCCCAGTTCCAGTACGGCTCCGACGAGCTTACT CGTTTCGTCCTCACGGACTCATCAGGGAGCCCGGTGATGTCTGCTCAAGC	pFC334 (AddGene #87846)
27	pLM1_95 B	F:TGGTCTCAGTTTCAGAGCTAGGCCAACATG R:CGGTCTCTGGCCAAAAAGCACCGACTCGGTGCC	pLM1_91

3

Seq. ID	Description	Sequence (5′ to 3′)	Template
28	pLM1_95 C	F:CGGTCTCAGGAGGAGCCAAGAGCGGATTCCTCAGTCTCGTACGTCTC R:TGGTCTCAGGCCGGCATGGTCCCAG	pFC334 (AddGene #87846)
29	pLM1_90 A	F:CGGTCTCTAGCGGCGTAAGCTCCCTAATTGGCCC R:CGGTCTCTAAACCCTGCCCTTCGCCTGGGACAGACGAGCTTACTCGTTTCGTCCT	pFC334 (AddGene #87846)
30	pLM1_90 B	F:TGGTCTCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCT R:CGGTCTCAGGAGGAGCCAAGAGCGGATTCCTCAGTCTCGTACGTCTC	pFC334 (AddGene #87846)
31	pLM1_113 removable *lacZ* A	F:CGGTCTCTAGCGGCGTAAGCTCCCTAATTGGCCC R:CGGTCTCATCGGTGATGTCTGCTCAAGCGG	pICH41308 (AddGene #87846)
32	pLM1_113 removable *lacZ* B	F:GAAGACTCCCGACGAGACCCAGCTGGCACGACAGGTTTC R:GAAGACAAAAACGGAGACCACAGCTTGTCTGTAAGCGGATG	pICH41308 (AddGene #47998)
33	pLM1_113 removable *lacZ* C	F:TGGTCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG R:CGGTCTCAGGAGGAGCCAAGAGCGGATTCCTCAGTCTCGTACGTCTC	pICH41308 (AddGene #87846)
34	pBD_1_10 Marker-free donor DNA for DsRed KO	F:GCTCTGTACAGTGACCGGTGACTC R:CAGGGTTTGAGAACTCCGATCTTAAATCC	pBD1_10
35	Marker-free donor DNA C*ACVS KO	F:CCCAGTATAAGGAATTCCCCTCGAGCTTGTCTGTGATTGCGT TTTTTCTAACACTTGTTGTTGCATCCGATCCGTCCCTACCAATTA TTGGTCATTGACAGACATGGAAGAGTGGAACAA R:CGCAGACAACGGCTATTTTGTCTTCATGCCGTTCAACCACCT CTTCAATGAGATGGTGCAGTCGCTTTGATGAAGGGTACTCGCC ATCCGTGTTGTTCCACTCTTCCATGTCTGTCAATG	overlapping oligonucleotides
36	sgRNA spacer insert DsRed KO for pLM-AMA15.0*	F:ATGGTCTCACCGA**ACTGGG**CTGATGAGTCCGTGAGGACGAAACGAG R:ATGGTCTCTAAAC**GGAGCCGTACTGGAACTGGG**GACGAGCTTACT CGTTTCGTCCTCACGGACTCA	overlapping oligonucleotides
37	sgRNA spacer insert C*ACVS KO for pLM-AMA15.0*	F:ATGGTCTCACCGA**CACTAC**CTGATGAGTCCGTGAGGACGAAACGAG R:ATGGTCTCTAAAC**ATAGGCTTCTCGGCCACTAC**ACGAGCTTACTCG TTTCGTCCTCACGGACTCA	overlapping oligonucleotides
38	*in vitro* T7-sgRNA-transcription template (C*ACVS KO)*	F:ATGTAATACGACTCACTATA**gTAGTGGCCGAGAAGCCTAT**GTTTCA GAGCTATGCTGGAAA R:GTTTCAGAGCTATGCTGGAAACAGCATAGCAAGTTGAAATAAGACTA GTTCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT	overlapping oligonucleotides
39	cPCR DsRed mutation validation	F:CACTCAACCCATGGTACGAGC R:GCGAGCCATCCCTTGATCTCG	DsRed.SKL (AddGene #154222)
40	cPCR MF-ACVS KO	F:CTTCAGTCGCCCAGATTCTCGAT R:GGCAGGATACCAATGGAACGGAGA	DS54468
41	Removable *lacZ* transcription unit receiver site for pLM-AMA-002	F:GAAGACTCTTAAGGAGCGAGACCCAGCTGGCACGACAGGTT R:GAAGACAATTAAAGCGGGAGACCACAGCTTGTCTGTAAGCGGA	pICH41308 (AddGene #47998)

Table S2. List of synthetic and PCR oligonucleotide primers created DNA fragments *lowercase "g" indicates the transcription site of the T7 *in vitro* transcription (is applies), and the 20 bp target sequence of sgRNA and 6 bp reverse sequence required for the HH ribosome marked in **bold.**

Created vector	Description	Cloned Part IDs or MoClo units	Recipient backbone vector
pZB0_21	P40s	1, 2, 3	pICH41295
pLM0_23	spCas9 direct fusion	4	pICH41258
pDL0_9	eGFP-NLS direct fusion	5	pICH41264
pLM0_40	spCas9 stop codon	6	pICH41308
pYN0_10	Ttif35	7	pICH41276
pLM0_12	PgpdA	8	pICH41295
pCP0_30	ergA (Pc22g15550)	9, 10	pICH41308
pLM0_11	amdS (ANIA_08777)	11-16	pICH41308
pZB0_20	TamdS	17	pICH41276
pYN0_9	Tact	18	pICH41276
pLM1_87	PKS17 5'flank	19	pICH47732
pLM1_88	PKS17 3'flank	20	pICH47772
pCP1_45	PgpdA-ergA-Tamds	pLM0_12, pCP0_30, pZB0_20	pICH47751
pLM1_82	P40s-spCas9-eGFP-Ttif35	pZB0_21, pLM0_23, pDL0_9, pYN0_10	pICH47742
pLM1_91	P-UmayU6-sgRNA-Tu6	21, 22	pICH47761
pLM1_92	P-ScereSNR52-sgRNA-Tu6	21, 23	pICH47761
pCP1_82	P-tRNA[Ser]-Pc-sgRNA-Tu6	24, 25	pICH47761
pLM1_95	P-gpdA-HH-sgRNA-HDV-Ttrpc	26, 27, 28	pICH47761
pLM1_90	P-gpdA-X-Ttrpc Non-sgRNA control	29, 30	pICH47761
pLM1_114	P40s-spCas9-Ttif35	pZB0_21, pLM0_40, pYN0_10	pICH47742
pLM1_113	P-gpda-removable *lacZ*-HDV-Ttrcp (FastAP treated backbone, assembly ended with ligation instead of restriction)	31, 32, 33	pICH47761
pBD1_10	MF Donor DNA vector DsRed KO	pLM0_12, pLM0_11, pYN0_9	pICH47751
pLM2_101	P-Umay, ergA, PKS17-flanking	pLM1_87, pLM1_82, pCP1_45, pLM1_91, pLM1_88, pICH49299	pICH50505 (alternative to pAGM4673)
pLM2_102	P-ScereSNR52, ergA, PKS17-flanking	pLM1_87, pLM1_82, pCP1_45, pLM1_92, pLM1_88, pICH49299	pICH50505 (alternative to pAGM4673)
pLM2_104	P-tRNA[Ser]-Pc, ergA, PKS17-flanking	pLM1_87, pLM1_82, pCP1_45, pCP1_82, pLM1_88, pICH49299	pICH50505 (alternative to pAGM4673)
pLM2_127	P-gpda, ergA, PKS17-flanking	pLM1_87, pLM1_82, pCP1_45, pLM1_95, pLM1_88, pICH49299	pICH50505 (alternative to pAGM4673)
pLM2_100	P-gpda (non-sgRNA control), ergA, PKS17-flanking	pLM1_87, pLM1_82, pCP1_45, pLM1_90, pLM1_88, pICH49299	pICH50505 (alternative to pAGM4673)
pLM2_135	dSpCas9-VPR-NLS, sgRNA transcription unit, ergA marker, penicillin BGC-flanking	pZB1_1, pLM1_100, pCP1_45, pLM1_113, pZB1_2, pICH41800	pICH50505 (alternative to pAGM4673)

pLM-AMA-002	MoClo entry vector compatible fungal shuttle vector, replaceable *lacZ* acceptor, phleomycin (bleo) marker	41	pLM-AMA-001
pLM-AMA-002_ P40S-spCas9- eGFP-NLS-Ttif35	fungal shuttle vector, carrying spCas9- eGFP-NLS transcription unit phleomycin (bleo) marker	pLM0_23, pDL0_9, pYN0_10	pLM-AMA-002
pLM-AMA-15_ DsRed_KO	CRISPR/Cas9 and sgRNA expressing vector, targeting DsRed ORF, phleomycin (bleo) and terbinafine (ergA) markers	36	pLM- AMA-15.0
pLM-AMA-15_ ACVS_KO	CRISPR/Cas9 and sgRNA expressing vector, targeting DsRed ORF, phleomycin (bleo) and terbinaf ine (ergA) markers	37	pLM- AMA-15.0

Table S3. List of vectors constructed in this study

CHAPTER 4

CRISPR-Based Transcriptional Activation Tool for Silent Genes in Filamentous Fungi

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Abstract

Filamentous fungi are historically known to be a rich reservoir of bioactive compounds that are applied in a myriad of fields ranging from crop protection to medicine. The surge of genomic data available shows that fungi remain an excellent source for new pharmaceuticals. However, most of the responsible biosynthetic gene clusters are transcriptionally silent under laboratory growth conditions. Therefore, generic strategies for activation of these clusters are required. Here, we present a genome-editing-free, transcriptional regulation tool for filamentous fungi, based on the CRISPR activation (CRISPRa) methodology. Herein, a nuclease-defective mutant of Cas9 (dCas9) was fused to a highly active tripartite activator VP64-p65-Rta (VPR) to allow for sgRNA directed targeted gene regulation. dCas9-VPR was introduced, together with an easy to use sgRNA "plug-and-play" module, into a non-integrative AMA1-vector, which is compatible with several filamentous fungal species. To demonstrate its potential, this vector was used to transcriptionally activate a fluorescent reporter gene under the control of the *penDE* core promoter in *Penicillium rubens*. Subsequently, we activated the transcriptionally silent, native *P. rubens* macrophorin biosynthetic gene cluster by targeting dCas9-VPR to the promoter region of the transcription factor *macR*. This resulted in the production of antimicrobial macrophorins. This CRISPRa technology can be used for the rapid and convenient activation of silent fungal biosynthetic gene clusters, and thereby aid in the identification of novel compounds such as antimicrobials.

Keywords: CRISPRa, dCas9, filamentous fungi, *Penicillium rubens*, secondary metabolites, biosynthetic gene clusters

Introduction

Fungi are amongst the most proliferous producers of secondary metabolites (SMs). These molecules, while not intrinsically required for survival, provide a biological advantage to their host[1]. Many fungal SMs are beneficial to humankind and have a wide range of applications in human and animal healthcare (e.g. as antibiotics or immunosuppressants), food, agricultural and industrial sectors[2,3]. On the other hand, SMs can be toxic and some SMs contribute to the pathogenicity of fungi while others contaminate food and crops[4]. Genes involved in secondary metabolism are often arranged in clusters, so-called biosynthetic gene clusters (BGCs), and these are typically regulated by pathway-specific transcription factors. As more fungal genomes, and bioinformatics tools and databases (e.g. fungal antiSMASH[5], MIBiG[6]) have become available for the prediction, annotation and prioritization of fungal BGCs, it has become clear that filamentous fungi have an even larger biosynthetic potential than previously anticipated.

Most of the BGCs are transcriptionally silent under laboratory growth conditions, therefore products of these clusters remain elusive[7]. Various methodologies have been developed for the activation of silent BGCs, including manipulation of both BGC specific as well as global transcriptional regulators, promoter-exchange, and heterologous expression in suitable host systems.[8] Marker-free genome editing remains challenging, and with only a limited number of fungal selection markers available, extensive genome manipulations is a laborious task.

The bacterial CRISPR/Cas systems have emerged as versatile biotechnological tools[9,10], and next to genome editing it can provide a promising alternative approach for transcriptional activation in fungi. CRISPR/Cas systems consist of only two components; a Cas nuclease and a programmable guide RNA. In case of the popular Cas9 system from *Streptococcus pyogenes* the protein can be guided to a genomic locus in a sequence-specific manner, using a single guide RNA (sgRNA) which consist of a short targeting crRNA sequence and the scaffold tracrRNA sequence. Methods for Cas9-based genome editing have been established in various filamentous fungal species[11,12], including the industrially

relevant fungi *Penicillium rubens*[13,14] (formerly identified as *P. chrysogenum*[15]). Cas9 and sgRNA delivery strategies include vector-based expression or genomic integration of transcriptional units encoding Cas9 and sgRNA. Alternatively, only Cas9 is expressed and the sgRNA is provided by a transformation of *in vitro* transcribed RNA, or both Cas9 and sgRNA are provided as pre-assembled ribonucleoprotein complexes (RNPs). The CRISPR/Cas9 genome editing tools established in filamentous fungi can edit the genome at a single as well as at multiple locations, and have effectively been applied in industrial fungi to improve compound production[11,12].

Beyond genome editing, CRISPR/Cas can be used as a platform for RNA guided DNA-protein interactions, and thereby deliver various effector domains to a specific genomic location. By introducing point mutations into the two nucleolytic domains, nuclease deficient versions of Cas9, called dead Cas9 (dCas9), were created[16]. Because dCas9 binds in a sequence-specific manner, but does not cleave DNA, it can be used for transcriptional regulation[17,18], epigenome editing[19,20], visualization of specific genomic loci[21] and base editing[22] in various eukaryotic species. For CRISPR/Cas mediated transcriptional activation (CRISPRa) several activating effector domains have been fused to dCas9.[23,24] The often-used VPR system consists of a three-component complex, four copies of the herpes simplex VP16 transactivation domain, the transactivation domain of nuclear factor kappa B, and Epstein-Barr virus R transactivator, VP64-p65-Rta, respectively[16]. dCas-VPR fusions have been successfully employed for upregulation of reporter and/or endogenous genes in mammalian cells[16], in diploid[25] and polyploid[26] *Saccharomyces cerevisiae*, *Yarrowia lipolytica*[27], *Candida albicans*[28], and most recently also in the filamentous fungus *Aspergillus nidulans*[29].

Here we report on the implementation of a dCas9-VPR-based, genome editing free system for transcriptional activation system in the filamentous fungus *Penicillium rubens*. We successfully utilized the CRISPRa tool to activate the cryptic macrophorin BGC, resulting in production of compounds with antimicrobial activity.

Results

Construction of a fungal CRISPRa tool

CRISPR/Cas mediated gene expression activation (CRISPRa) requires a catalytically dead CRISPR-associated protein (dCas) fused to an activation domain, as well as a sgRNA to guide it to the desired locus. Here, the widely utilized fusion of dCas9 from *Streptococcus pyogenes* to the tripartite activator, VP64-p65-Rta (VPR)[16] was selected for activation. For easy implementation of CRISPRa in a broad range of filamentous fungi, we constructed an AMA1-based vector for expression of the NLS tagged dSpCas9-VPR under the 40S ribosomal protein S8 promoter (P*40S*) (Fig. 1a). The AMA1 sequence -originally isolated from *A. nidulans*- allows for autonomous vector replication in several filamentous fungal species[30,31], and is often employed for Cas9 and sgRNA expression in gene-editing approaches in these organisms[11,13,32,33]. The AMA1 vector was also used to supply the sgRNA, establishing CRISPRa after a single transformation. The sgRNA was expressed from the constitutive *gpdA* promoter and flanked by hammerhead (HH) and hepatitis delta virus (HDV) ribozymes to ensure defined ends for sgRNA processing and optimal functionality (Fig. 1a)[34].

Target specificity is determined by the sgRNA, thus by exchanging the sgRNA sequence different genes can be targeted for upregulation. To enable convenient and efficient exchange of sgRNA target sequences a sgRNA "plug-and-play" module was introduced into the AMA1 shuttle vector to facilitate cloning steps in *Escherichia coli* (Fig. 1a-b). This vector, which is the parent to all sgRNA expressing vectors, is called pLM-AMA18.0-dCas9-VPR (referred to as pAMA18.0) and also functions as a negative (non-targeting sgRNA) control. The sgRNA "plug-and-play" module works as follows; the chimeric sgRNA backbone sequence and the HDV ribozyme are already supplied on the AMA1-vector together with a *lacZ* gene flanked by BsaI restriction sites. The 20 bp spacer sequence defining the genomic target is supplied on a separate dsDNA molecule, together with the hammerhead ribozyme (HH) which includes the necessary 6 bp inverted repeat of the 5'-end of the spacer to complete the HH cleavage site. This dsDNA molecule can simply be created by PCR using two overlapping

oligonucleotides (Fig. 1b) or alternatively ordered as chemically synthetized dsDNA. The fragment can then be inserted into pAMA18.0 using the Golden Gate cloning and the BsaI restriction sites[35]. As this removes the *lacZ* gene, positive bacterial clones can easily be detected with blue-white screening. After positive sequence verification and vector extraction, the created CRISPRa vector can be introduced into the filamentous fungi of choice (Fig. 1b).

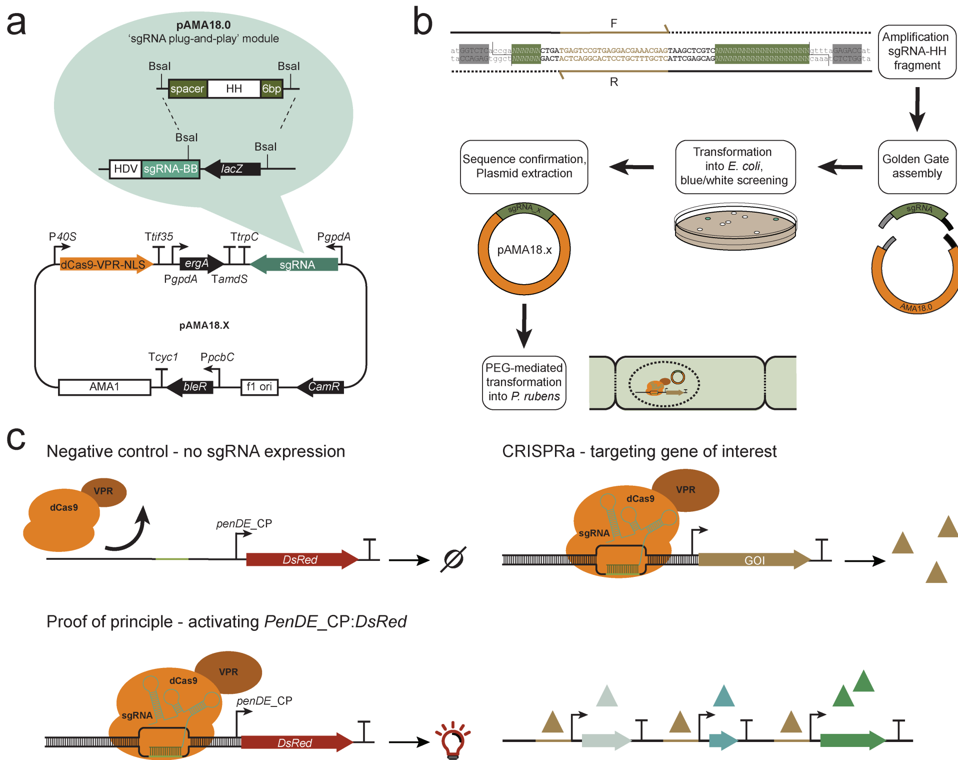

Figure 1 Overview of the programmable CRISPR/Cas-based transcriptional activation system implemented in *P. rubens*. (**a**) Schematic representation of the pAMA18.X-vector encoding the components of the CRISPR/Cas activation system, namely the dCas9-VPR and the ribozyme self-cleaved sgRNA. pAMA18.0 is the parent vector of all sgRNA coding vectors and contains the sgRNA "plug-and-play" module which is highlighted. (**b**) Diagram depicting the cloning strategy for insertion of the PCR amplified sgRNA into pAMA18.0. (**c**) CRISPRa proof of principle. In the control strain carrying pAMA18.0 no sgRNAs are transcribed, so while dCas9-VPR is present it is not targeted to a specific locus and no transcriptional activation occurs. Correct targeting of the dCas9-VPR complex to the silent *penDE*-CP is leading to DsRed fluorescent protein expression and hence increased fluorescence. In the same fashion when dCas9-VPR is targeted to a promoter driving a gene of interest, this results in product formation. When the targeted promoter drives a transcriptional regulator this can result in activation/repression of multiple other genes, including entire BGCs.

Proof of principle – activating penDE-CP_DsRed

In order to test if expression of dCas9-VPR and the sgRNA from the CRISPRa vector could activate transcription of a silent gene, we targeted dCas9-VPR to the *penDE* core promoter (*penDE*-CP). The 200 bp long *penDE*-CP has previously been shown to be functional, but insufficient to drive expression on its own[36]. For easy visualization of CRISPR based transcriptional activation, the *penDE*-CP was set to drive *DsRed-T1-SKL*, a red fluorescent reporter gene with peroxisomal targeting signal (Fig. 1c). The *penDE*-CP_*DsRed* reporter unit was integrated into the penicillin-locus of the *P. rubens* DS68530 (Δpenicillin-BGC), utilizing CRISPR/Cas9 ribonucleoprotein (RNP) facilitated co-transformation[13,14] (Supp. Fig. S1a).

Different pAMA18.0 derived vectors (pAMA18.a-f) expressing sgRNAs targeting loci +1 to -118 bp relative to the transcription start site (TSS) of the *penDE*-CP (Fig. 2a, Supp. Fig. S2a, Supp. Table S1) were transformed into *P. rubens* DS68530_*penDE*-CP_*DsRed* and strains were analyzed using fluorescence microscopy (Fig. 2b). Increased fluorescence intensity was seen in strains transcribing *penDE*-sgRNA_c, _d, and _e but not in strains transcribing *penDE*-sgRNA_a, _b and _f. The DS68530_*penDE*-CP_*DsRed* strain carrying the pAMA18.0 negative control vector which did not express any sgRNA, showed only a minimal amount of fluorescence. DsRed expression was also evaluated using qPCR, showing the most efficient activation for *penDE*-sgRNA_c (Fig. 2c). These results confirm that activation of DsRed expression was CRISPRa dependent.

To assess the performance of the different sgRNA target sequences, the BioLector microbioreactor system was used with online monitoring of scattered light (biomass) and red fluorescence intensity (Fig. 2d, Supp. Fig. S3). The strength of *DsRed* activation by different sgRNAs was determined relative to biomass to avoid variance caused by small differences in growth. During the time interval of 0-40 h, DsRed/biomass values in CRISPRa strains were measured and compared to negative control strain carrying pAMA18.0 and the background fluorescence of the DS68530 parental strain. The pAMA18.c carrying strain showed the highest level of relative fluorescence and thus provided the most efficient activation compared to the non-sgRNA negative control (Fig. 2d). All other CRISPRa strains

show activation of *penDE*-CP_*DsRed*, with weakest activation in strains carrying pAMA18.a and pAMA18.b vectors.

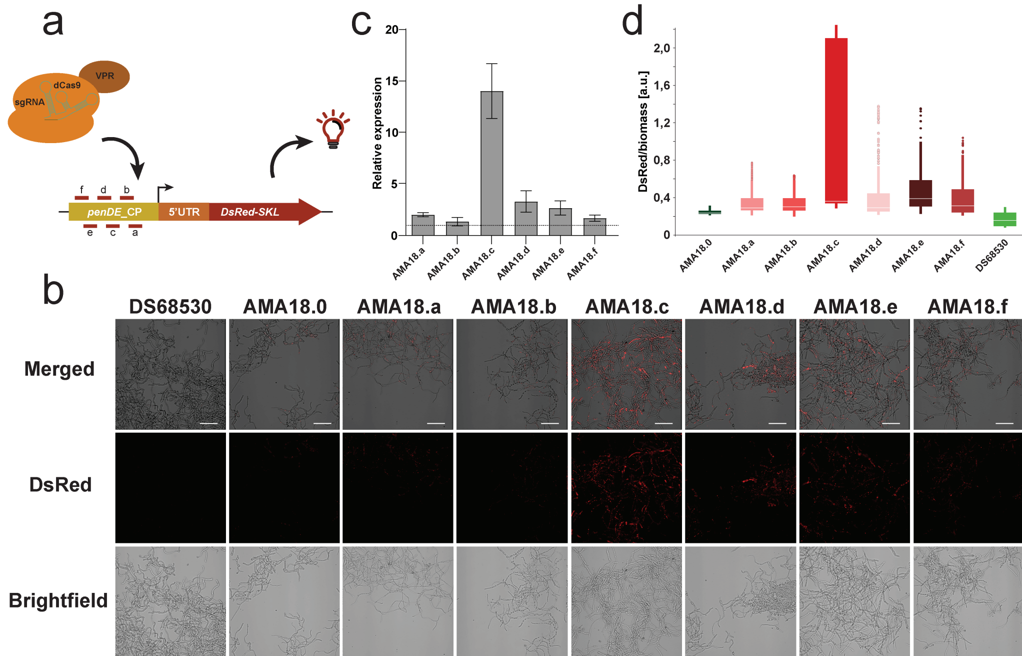

Figure 2 CRISPRa (dCas9-VPR) based activation of *penDE*-CP_*DsRed*. (**a**) Schematic representation of the *penDE*-CP upstream *DsRed*. The transcription start site (TSS) is indicated as a black arrow, short lines with letters indicate targeting sites of the sgRNAs. (**b**) Confocal fluorescence microscopy imaging of *DsRed* targeting CRISPRa strains and controls with no-sgRNA (AMA18.0) and without the *penDE*-CP_*DsRed* transcription unit (DS68530). Strains were grown for 5 days in liquid SMP media. Scale bars represent 50 µm. (**c**) qPCR analysis showing expression levels of *DsRed* in CRISPRa strains relative to strain carrying pAMA18.0 no-sgRNA negative control (dotted line) after 5 days of growth in SMP. (**d**) Development of DsRed/biomass over time during time window of 0-40 hours cultivation in the BioLector system. Data were obtained from 3 separate experiments, each consisting of 2-3 biological replicates; error bars show the standard deviation.

CRISPRa-based activation of the transcriptionally silent macrophorin gene cluster

Meroterpenoids represent a large family of natural compounds with diverse biological activities, such as the antimicrobial yanuthones found in *Aspergillus niger*[37,38]. Highly identical clusters have been found in *Penicillium* species[39]. These *Penicillium* BGCs contain an additional gene (*macJ*), which was shown in *Penicillium terrestris* to encode a terpene cyclase responsible for cyclization of linear yanuthones leading to production of diverse macrophorin analogs[39]. The putative *P. rubens* macrophorin BGC consists of 11 biosynthetic genes, namely *macA*-*J* and *macR* as a transcriptional regulator of the cluster (Fig. 3a-b).

Sequence alignment of the provisional sequence of *P. rubens macR* (*Pc16g00410)* to the *P. terrestris* LM2 *macR* coding sequence (*MF989995.1*) shows that the *P. rubens* sequence is predicted to have an additional intron leading to a premature stop codon. Without this intron, the *P. rubens macR* mRNA should produce a full-length product, similarly to *P. terrestris* LM2 *macR*. To test if *macR* codes for a functional protein we performed promoter replacement in *P. rubens* DS68530, substituting the promoter region of *macR* with the promoter of the *pcbC* (isopenicillin N synthase) gene (Supp. Fig. S1b), creating strain *macR*:OE. The resulting increase in *macR* transcription (Fig. 3c) led to the activation of the cryptic BGC (Fig. 3d-e) and the production of macrophorins (Fig. 3f-g, Supp. Table S2). We therefore conclude that *P. rubens macR* encodes for a functional transcription factor and that increased expression of *macR* leads to activation of the entire associated BGC. Moreover, activation of this BGC leads to production of macrophorin-like compounds (Supp. Table S2).

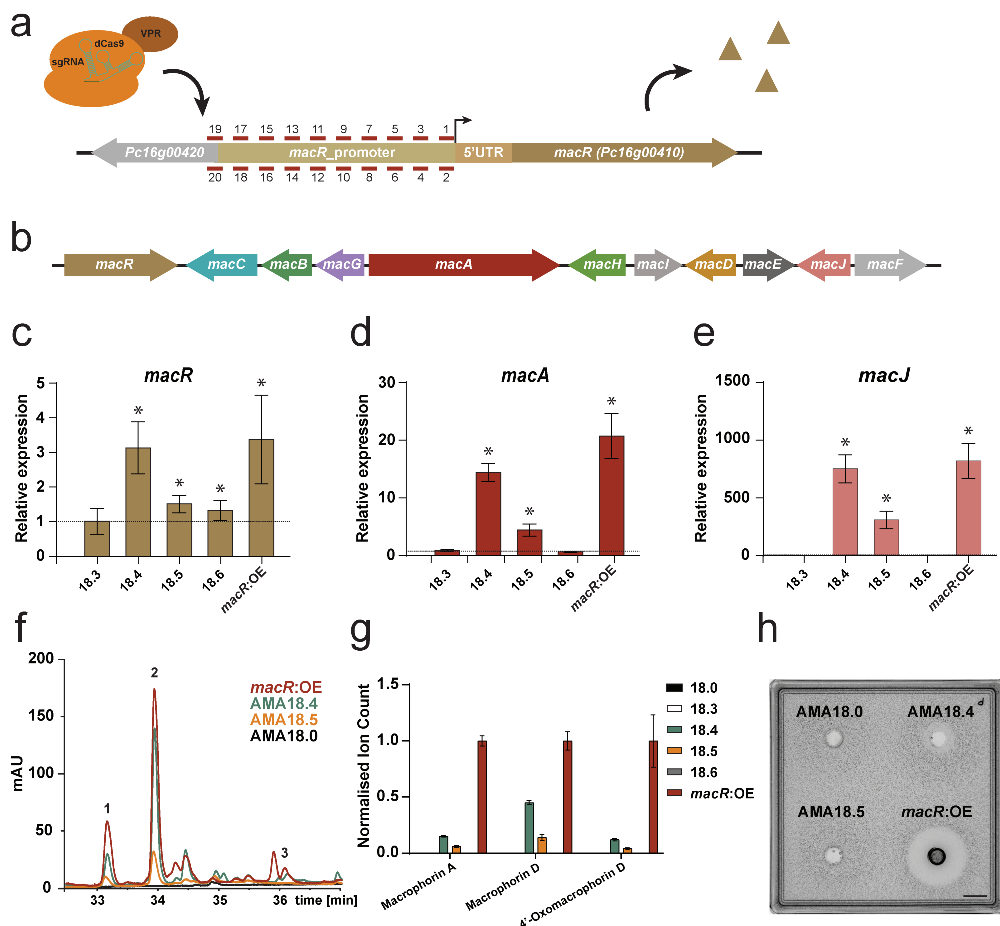

Figure 3 CRISPRa activation of the macrophorin BGC. (a) Schematic representation of the macR promoter region. The location of the putative transcription start site (TSS) is indicated as a black arrow, short lines with number indicate sgRNAs targeting sites. (b) Schematic representation of the macrophorin BGC. qPCR analysis showing expression of macR (c) macA (d) and macJ (e) in the CRISPRa and macR:OE strains, relative to the strain carrying the pAMA18.0 vector (non-target control) after 5 days of growth in SMP medium. Error bars indicate the standard error of the mean of three biological replicates with two technical duplicates, and (*) indicates significant up-regulation (Student's-test p-value <0.05). (f) LC-MS UV-VIS chromatogram (λ=700 nm) of hyphae extracts of CRISPRa and macR:OE strains representing macrophorin A (1), macrophorin D (2) and 4'-oxomacrophorin D (3). (g) LC-MS analysis of macrophorin related compounds in hyphae extracts of CRISPRa and *macR*:OE strains. Error bars indicate the standard error of the mean of three biological replicates with two technical duplicates. (h) Bioassay to detect (macrophorin related) antimicrobial activity against *Micrococcus luteus* in the supernatant of indicated strains grown for 5 days in liquid SMP medium. The supernatant was concentrated 10-times and 100 µl was loaded in a well in top agar containing *M. luteus* at OD600 = 0.0125. Scale bar represents 10 mm.

Sanger sequencing data of cDNA obtained from the *macR*:OE strain showed 2 introns in *P. rubens macR mRNA and* no pre-mature stop codon, in line with the coding sequence of *macR* of *P. terrestris* (*MF989995.1*) and the homologous *yanR* (*ASPNIDRAFT_44961*) of *A. niger*. It therefore seems likely that the third intron in the provisional *P. rubens macR* sequence is wrongly predicted, and *P. rubens* is capable of producing, not only functional, but also full-length MacR. Additionally, a mutation (cDNA 2611C>T, P776S) mutation was identified in the ORF of *macR*. The effect of this mutation was not further investigated as *macR* remained capable of transcriptional activation. The sequence of *P. rubens* DS68530 *macR* cDNA can be found in Supp. Note S1.

Since the *P. rubens* macrophorin BGC is silent under our growth conditions (Secondary Metabolite Producing [SMP] medium, 25°C)[40,41], it was selected for activation by CRISPRa. As no TSS is known for *macR*, 20 sgRNAs (*MacR*-sgRNA_1-20) were designed to target the entire 547 bp long, native promoter (Fig. 3a, Supp. Table S1, Supp. Fig. S2b). The *macR* targeting CRISPRa strains and the *macR*:OE positive control, were grown on SMP-agar for 10 days after which secondary metabolites were extracted from representative agar plugs, and analyzed by LC-MS (Supp. Table S3). As expected, no macrophorin production was observed in the strain carrying the pAMA18.0 negative control with no sgRNA insert. Strains expressing *MacR*-sgRNA_4 and *MacR*-sgRNA_5 showed production of compounds with masses corresponding to macrophorin A (361.24 m/z $[M+H]^+$), macrophorin D (505.28 m/z $[M+H]^+$) and 4′- oxomacrophorin D (503.26 m/z $[M+H]^+$) (Supp. Table S2). None of the other CRISPRa strains exhibited macrophorin production.

Fungal strains carrying vector pAMA18.3-6 and pAMA18.0 (no sgRNA control) were further investigated by qPCR (Fig. 3c-e) and metabolite profiling (Fig. 3f-g). Strains expressing *MacR*-sgRNA_4 and sgRNA_5 were selected as these sgRNAs showed activated macrophorin production (Supp. Table S3). Although strains carrying *MacR*-sgRNA_3 and sgRNA_6 did not show macrophorin production these strains were also investigated further, as these sgRNAs target the *macR* promoter region in close proximity to the successfully activating *MacR*-

sgRNA_4 and sgRNA_5, but on the opposite strand of the DNA. As expected, strains carrying the pAMA18.4 or pAMA18.5 CRISPRa vector showed an increase in *macR* expression compared to the pAMA18.0 control, further confirming CRISPRa dependent transcriptional activation (Fig. 3c). The increase in *macR* expression resulted in transcriptional activation of the macrophorin BGC as exemplified by increased levels of *macA* (polyketide synthase) (Fig. 3d) and *macJ* (proposed terpene cyclase[39]) mRNA (Fig. 3e), that respectively encode the first and last enzymes in the macrophorin biosynthesis pathway[39].

In the strain carrying the pAMA18.4 vector, levels of transcriptional activation were comparable to those in the positive control *macR*:OE while strain carrying vector pAMA18.5 showed a ~3-fold lower transcription compared to this control, for all genes investigated (Fig. 3 c-e). No increased expression of *macR*, *macA* or *macJ* was observed for the strain carrying pAMA18.3. In the strain carrying vector pAMA18.6, a slight upregulation of *macR* was observed but this did not result in induction of *macA* and *macJ* (Fig. 3 c-e). In line with this, the strain carrying pAMA18.5 produced lower amounts of the examined macrophorin related metabolites compared to the strain with pAMA18.4 (Fig. 3 f-g). However, while qPCR analysis showed similar mRNA levels between the *macR*:OE and pAMA18.4 strains, compound production for macrophorin A and 4′-oxomacrophorin D was lower in AMA18.4 compared to the *macR*:OE strain, reaching 15% and 13% respectively. Strain AMA18.4 reached highest production for macrophorin D at ~38% of the ion intensity measured in *macR*:OE.

As the related yanuthones produced by *A. niger* display antimicrobial activity against gram positive bacteria[42], we analyzed the activity of our macrophorin producing *Penicillium* strains against *Micrococcus luteus* using the agar diffusion method. The transformed parent strain *P. rubens* DS68530 does not contain the penicillin BGC, and consequently does not produce compounds inhibiting the growth of *M. luteus*. We observed a clearance zone around concentrated supernatant from the *macR*:OE strain grown for 5 days in SMP medium, and to a lesser extent also around that of the AMA18.4 strain, but not that of the control (AMA18.0) or the AMA18.5 strain (Fig. 3h). This indicates that

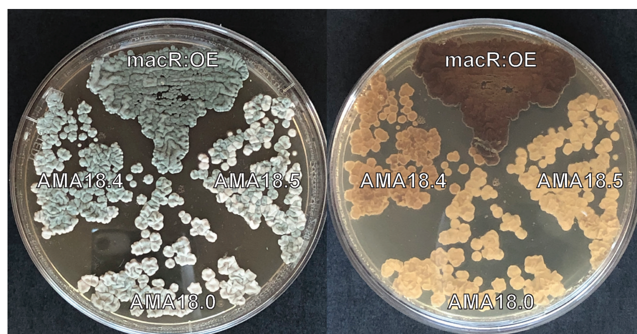

Figure 4. Dark pigmentation of hyphae due to *macR* overexpression in *Penicillium rubens* DS68530 *macR*:OE and in the CRISPRa pAMA18.4 vector carrying strain after 5 days of cultivation on R-agar, compared to AMA18.0 strain carrying pAMA18.0 (no sgRNA) negative control.

the macrophorins produced by *P. rubens* are indeed bioactive against Gram-positive bacteria, and CRISPRa dependent activation of the BGC is sufficient to induce antimicrobial activity.

Interestingly, we observed a dark brownish pigmentation of the hyphae of the *macR*:OE strain after 5 days of cultivation on R-agar and SMP-agar as well as on day 1 in SMP liquid medium. The strain carrying the CRISPRa vector pAMA18.4 displayed a milder coloration compared to the colorless hyphae of the parent strain (Fig. 4). Color formation in these *macR* over-expression strains was not investigated further.

Discussion

In this work, we report the application of dCas9-VPR based CRISPRa in the ascomycetous filamentous fungus *Penicillium rubens*. While *Penicillium* is acclaimed for its production of ß-lactam antibiotics, it harbors many more BGCs of which a substantial portion remain uncharacterized[43]. CRISPRa systems have been established in many model organisms as an ideal technology for transcriptional regulation and could aid in activating these often silent BGCs to facilitate characterization.

In our approach dCas9-VPR and the sgRNA are episomally encoded on the same AMA1-based vector, hence a single transformation with a single vector is enough to establish CRISPR-based transcriptional activation in *Penicillium*, without the need for genome engineering of the host organism. Moreover, because AMA1 supports autonomous vector replication in several filamentous

fungal species[30,31], and as we use established fungal promoters, terminators, and ribozyme based sgRNA processing, we expect the vector to be transferable to other fungal species. The sgRNA "plug-and-play" module of our CRISPRa vector combines Golden Gate cloning approach with blue/white screening. This allows for convenient cloning of new sgRNAs into the vector, reducing experimental time. This is especially important since general criteria for successful sgRNA design are difficult to define, and empiric testing of sgRNAs for each promoter region of interest remains necessary. Due to the ease of cloning our AMA1 vector, this CRISPRa technology could potentially be implemented in connection with larger scale fungal protoplast transformations using microtiter plates[44], for example in combination with deploying multiple, separate sgRNA processing vectors in one transformation.[45]

To assess the CRISPRa vector for activation of transcriptionally silent promoter activation, we integrated a *penDE* core promoter driven *DsRed* gene into the penicillin-locus of *P. rubens* DS68530 (Δpenicillin-BGC). This *penDE*-CP was selected because it has been reported previously to be insufficient to express the fluorescent reporter on its own, instead depending on the presence of a synthetic transcription factor[36]. Fluorescence microscopy showed a clear increase in fluorescence with 3 out of 6 sgRNAs tested, compared to a non-sgRNA expressing control (Fig. 2b). Quantification of fluorescence using a BioLector microbioreactor showed increased fluorescence for 6 out of 6 sgRNA used, showing weak activation for *penDE*-sgRNA_a and *penDE*-sgRNA_b sgRNAs, and, in line with fluorescence microscopy results, *penDE*-sgRNA_c standing out as the most efficient activator (Fig. 2d). The discrepancy between fluorescence microscopy and the BioLector results could possibly be explained by a higher sensitivity of the BioLector, different cultivation method and time points (day 5 of shake-flask cultures for microscopy, average fluorescent during the first 40 hours for the BioLector cultivations).

In *A. niger*, Roux and co-workers observed that dCas9-VPR mediated activation of a mCherry fluorescent reporter fused to the transcriptionally silent *Parastagonospora nodorum elcA* promoter was stronger with sgRNAs targeting

closer to the start codon, in a window of 162-342 bp upstream of the ATG [29]. We target a region 106-170 bp upstream the start codon ATG (32-96 bp upstream the TSS) and observe the highest activation with *penDE*-sgRNA_c targeting 129 bp upstream the ATG, and the least with *penDE*-sgRNA_a and _b (not detectable by microcopy) targeting closer to the start codon. We thus do not see the same trend – stronger CRISPRa for sgRNAs targeting closer to the start codon – however we already target a window closer to the ATG compared to Roux *et al.*[29] This exemplifies that it remains difficult to define an optimal targeting conditions, and ideally several sgRNAs should be tested when establishing CRISPRa for a new promoter. In line with what previously was reported for *S. cerevisiae*, we did not observe an effect on CRISPRa efficiency when targeting the plus or minus strand.[46]

To show our CRISPRa system can upregulate an entire silent BGC in *P. rubens* and induce metabolite production, we targeted the *macR* transcription factor of the endogenous macrophorin biosynthesis cluster. Macrophorins are a member of the meroterpenoids, a family of natural compounds which also include, for example, the antimicrobial yanuthones produced by *A. niger*[37,38]. Homologous macrophorin BGC have been identified in *Penicillium* species, and *P. terrestris* has been shown to produce macrophorins, through the cyclization of yanuthones[39]. Out of the 20 sgRNAs tested, two resulted in transcriptional activation of the macrophorin BGC (through the activation of transcriptional factor *macR*) (Fig. 3c-d) and secondary metabolite production (Fig. 3f-g). Although it is impossible to distinguish macrophorins and yanuthones with the method used as they have the same molecular formula, activation of the *macJ* terpene cyclase should lead to cyclic macrophorins.[39] Additionally, we could show that the supernatant of the CRISPRa activated strain grown five days in SMP media exhibited antimicrobial activity against the Gram-positive bacterium *M. luteus* (Fig. 3h). This clearly shows that our dCas9-VPR vector is capable of awakening silent BGCs in *Penicillium* and that the method can aid in product identification and characterization. It should be noted that exchanging the native *macR* promoter with the *pcbC* promoter resulted in higher compound production (Fig.

3g). It might therefore be beneficial to perform promoter exchange for high level production of interesting compounds identified using the CRISPRa technology. A possible explanation for why a larger proportion of the sgRNAs targeting *penDE*-CP (6/6) lead to transcriptional activation compared to *macR* (2/20) may be that the CP is free from most of its native regulatory elements, reducing chances of interference with the binding of the dCas9-VPR regulator. A limiting factor for this way of BGC activation is the need to identify a positive regulator for the cluster, which might not always be known. However, bioinformatics tools like antiSMASH[47] or CASSIS[48] could aid by identifying candidate regulators.

Recently, dCas12a (previously Cpf1), from *Lachnospiraceae bacterium* (dLbCas12a) or *Acidaminococcus* sp. (dAsCas12a), has become a popular alternative to dCas9 for gene regulation[49,50]. The Cas12a system has been popularized due to its ease of multiplexing; dCas12a uses smaller guide RNAs and is capable of processing these from a longer precursor CRISPR RNA[51]. Recent literature shows processing of 20 crRNA from a single precursor and simultaneous upregulation of 10 genes by dCas12a fused to a combination of the p65 activation domain together with the Heat shock factor 1 in human embryonic kidney (HEK) 293T cells, exemplifying the potential of multiplex gene regulation using dCas12a[52]. A potential drawback for using dCas12a in fungi is the low activity at temperatures below 28°C, while most fungal species grow optimally at temperatures between 25°C and 30°C. However, Roux and co-workers recently engineered an temperature tolerant Cas12a mutant (dLbCas12a^{D156R}-VPR), which was successfully employed for CRISPRa mediated gene activation in *A. nidulans* at 25°C.[29] While dCas12a is an attractive choice when aiming to upregulate multiple genes simultaneously, for single target activation dCas9-VPR is still a good option. We got significant upregulation of an entire BGC using a single sgRNA targeting the TF of the BGC. For dLbCas12a based upregulation in *A. nidulans* (the unmutated dLbCas12a grown at 37°C) multiple crRNAs were required for gene activation[29]. Another consideration when choosing a system is the different PAM requirement, NGG for (d)Cas9 and TTTN for (d)Cas12a. Depending on PAM availability in the genome one or the other could be preferable.

In conclusion we demonstrated that CRISPRa, specifically AMA1 vector-based expression of a dCas9-VPR fusion, can be used for the transcriptional activation of silent BGCs in *P. rubens*. We anticipate that the CRISPRa tool presented here can be widely used to awaken cryptic BGC in filamentous fungal species and thereby aid in the discovery of novel bioactive secondary metabolites.

Materials and methods

Chemicals, reagents and oligodeoxyribonucleotides

All medium components and chemicals were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands) or Merck (Darmstadt, Germany). Oligodeoxyribonucleotide primers (Supp. Table S4) were obtained from Merck. Enzymes were obtained from Thermo Fisher Scientific (Waltham, MA) unless otherwise stated. For design of nucleic acid constructs, *in-silico* restriction cloning, Gibson Assembly and inspection of Sanger sequencing results, SnapGene (GSL Biotech) was used.

Vector construction

The Golden Gate technology based Modular Cloning (MoClo) system[53] using Type IIS BpiI and BsaI restriction enzymes were employed for the construction of all vectors unless stated otherwise. Constructed vectors with their destination vectors and corresponding PCR fragments or DNA donor vectors can be found in Supp. Table S5. PCR amplifications were conducted using KAPA HiFi HotStart ReadyMix (Roche Diagnostic, Switzerland) according to the instructions of the manufacturer. Internal BsaI, BpiI recognition sites were removed for MoClo compatibility.

dCas9-2xNLS-VPR was amplified from two different sources. NLS-VPR was amplified from pAG414GPD-dCas9-VPR template (AddGene ID #63801)[16] and dCas9-NLS was amplified with adding D10A, D839A, H840A and N863A modifications from synthetic spCas9 pYTK036 template, provided as part of the Yeast MoClo Toolkit (AddGene ID #65143)[54]. DNA sequence of the created dCas9-VPR fusion can be found in Supp. Note S2.

The HH and HDV ribozyme based “Plug-and-Play” sgRNA transcription unit was amplified in three parts, where the *gpdA* promoter and the *trpC* terminator together with the HDV self-cleaving sequence were amplified from vector pFC334 (AddGene ID #87846)[33] and *LacZ alpha* gene was amplified from the MoClo ToolKit vector pICH41308 (AddGene ID #47998)[53]. The promoter of 40S ribosomal protein S8 of *A. nidulans* (*AN0465.2*, referred to as *40S*) and the *tif35* terminator of *P. rubens Pc22g19890,* as well as the transcription unit *penDE*-CP-*DsRed*-*SKL*-T*Act*, were amplified from pVE2_10 (AddGene ID #154228)[36]. The terbinafine selection marker as P*gpdA*-*ergA*-T*amdS* transcription unit was amplified from pCP1_45[41]. The promoter of *pcbC* (*Pc21g21380*, IPNS) was amplified from pVE2_19 (AddGene ID #154241)[36], adding 80 bp long flanking regions for homologous recombination. The phleomycin selection marker was amplified from pDSM-JAK-109[55] providing the P*pcbC*-*ble*-T*CYC1* transcription unit, adding 80 bp flanking regions for homologous recombination (Supp. Fig. S1b, Supp. Table S6).

Our autonomously replicating shuttle vector, carrying the AMA1 sequence, was based on pDSM-JAK-109[55] where the P*gpdA*-*DsRed*-*SKL*-T*penDE* transcriptional unit was removed using the BspTI and NotI restriction enzymes. The linear vector was treated with the Klenow Fragment and ligated to the circular vector using the T4 DNA Ligase according to the instructions of the manufacturer, creating a new AMA1 vector without *DsRed* expression. In order to create the CRISPRa vector, this vector was linearized with BspTI and was assembled by Gibson Cloning using PCR fragments G1, G2 G3 (Supp. Table S5) carrying a terbinafine selection marker, dCas9-VPR and the sgRNA transcription unit respectively. CRISPRa vector pLM-AMA18.0 is deposited to AddGene under ID #138945. Parallel with this work a catalytically active SpCas9 expressing vector was also established (pLM-AMA15.0 AddGene ID #138944) and utilized for genome editing [manuscript in preparation].

sgRNA target design and cloning

Promoter sequences were analyzed with CCTop[56] for possible CRISPR RNA guides

with the following limitations: protospacer adjacent motif (PAM): NGG, target sequence length 20 bp, core length 12 bp, mismatches taken into account for prediction in core sequence 2, number of total mismatches 4 and using *P. rubens Wisconsin 54-1255* as the reference genome. Predicted protospacers were manually curated for minimizing off-target effects and selecting high CRISPRater[57] scores. Primers were designed to create 89 bp long dsDNA inserts by PCR, containing the unique 20 bp spacer sequence, the hammerhead ribozyme, the 6 bp inverted repeat of the 5'-end of the spacer sequence and the BsaI type II restriction enzyme recognition sites.

For cloning the inserts into the vector pAMA18.0 a modified MoClo protocol[53] was used, using FastDigest BsaI (Thermo Fisher Scientific, Waltham, MA) restriction enzyme with an initial 10 min digestion, 50 cycles of digestion and ligation (37°C for 2 min, 16°C for 5 min), followed by a final digestion step and a heat inactivation step. Correctly assembled vectors were identified with blue-white screening and confirmed by sequencing. After positive sequence verification and vector extraction, the created pAMA18.X (where X stands for the sgRNA ID) CRISPRa vector was introduced into the fungal strain of choice (DS68530_*penDE*-CP_*DsRed* or DS68530) creating the CRISPRa fungal strain AMA18.X (Fig. 1b, Supp. Table S6).

Fungal strains and transformation

P. rubens strain DS68530[40] (Δpenicillin-BGC, Δ*hdfA*, derived from DS17690) was kindly provided by Centrient Pharmaceuticals (former DSM Sinochem Pharmaceuticals, Netherlands). Protoplasts of *P. rubens* were obtained 48 hours post spore seeding in YGG medium and transformed using the methods and media as described previously[14].

Mycelium was collected by centrifugation at 4000 x g for 8 min at 4°C. The pellet was resuspended in 50 ml KC solution (60 g/l KCl; 2 g/l citric acid; pH set to 6.2). After a second round of centrifugation, the pellet was resuspended in 18 ml KC solution and moved to sterile 100 ml shake flask. The mycelium solution was supplemented with 25 mg/ml Glucanex Lysing Enzyme

from *Trichoderma harzianum* (Sigma-Aldrich) and incubated at 25°C and 120 RPM for 90 min. Successful protoplast formation was confirmed by microscopy. Protoplast solution was moved to a sterile falcon tube and was kept on ice when possible. Protoplast were diluted to 50 ml using KC buffer and pelleted by centrifugation at 2770 x g for 5 min at 4°C (same settings were used in all subsequent centrifugation steps). Protoplast pellets were resuspended in 25 ml KC buffer followed by addition of 25 ml STC buffer (219 g/l sorbitol, 5.5 g/l $CaCl_2$, 10 mM Tris-HCl pH 7.5; pH set to 7.5 8.0). After centrifugation, pelleted protoplasts were resuspended in 50 ml STC and counted by microscopy using a counting chamber. After centrifugation protoplasts were resuspended in STC to obtain 2 x 10^7 protoplasts/ml (approximately 1 - 5 ml). These protoplasts were used fresh, or stored at 80°C in 10% (w/v) PVP-40 (Polyvinylpyrrolidone, Sigma-Aldrich) dissolved in STC as a cryopreservation buffer.

Protoplasts were transformed using PEG-mediated transformation[14]. In short, 200 µl protoplast solution (~2 x 10^7 protoplasts/ml) was added to a sterile 12-ml Greiner tube on ice, and were mixed with 1 - 8 µg DNA (in maximum 50 µl) and 200 µl 20% PEG-4000 solution (33 ml 60% PEG-4000; 67 ml STC buffer; 109.5 g sorbitol; 5 ml 1 M TRIS-HCl butter pH 7.5; in final volume of 250 ml). Protoplasts were incubated on ice for 30 min. Tubes were supplemented with 1.5 ml 60% PEG-4000 solution (60 g PEG-4000 dissolved in 40 ml H_2O by heating in a microwave, 1.0 ml 1 M Tris-HCl pH 7.5; 5.0 ml 1 M $CaCl_2$ in a total volume of 100 ml) and were homogenized completely by rotating the tube for 2 min. The tubes were placed in a 25°C incubator for 25 min. 1.2 M sorbitol was added to a total of 11 ml, and protoplasts were pelleted by centrifugation at 2770 x g for 5 min at 25°C. Protoplasts were carefully resuspended in 1 ml 1.2 M sorbitol and 100, 200 and 300 ul was plated on solid transformation medium.

When transforming the CRISPRa AMA1 vectors, total DNA did not exceed 1 µg. For Cas9-mediated genome editing of *macR*:OE and *penDE*-CP_*DsRed* strains, the appropriate Cas9 RNP mixtures were added. Using CRISPR/Cas9 ribonucleoprotein (RNP) facilitated co-transformation[13,36], marker free DNA was delivered in 10:1 molar ratio compared to the fungal marker, not exceeding 8 µg

total DNA. Synthetized sgRNAs were prepared using MEGAscript T7 Transcription Kit (Thermo Fisher Scientific, Waltham, MA) from PCR generated DNA templates, and the Cas9 protein was overexpressed in *E. coli* T7 Express *lysY* from pET28a/Cas9-Cys (AddGene ID #53261). For each transformation, separate Cas9 RNP mixtures were formed by mixing 27 µg Cas9 protein (up to 15 µl), 4 µl of synthetized sgRNA; 35 µl 2x STC and 30 µl 10x Cas9 activity buffer (HEPES 4.76 g/l; KCl 11.18 g/l; EDTA 0.029 g/l; $MgCl_2$ x 7 H_2O 2.03 g/l; pH set to 7.5; DTT 0.08 g/l).

Media and culture conditions

Solid transformation medium was prepared using SAG solid medium (Sucrose 375 g/l: Agar 15 g/l; Glucose Monohydrate 10 g/l) supplemented, in this order, with 4 ml/l Trace Element Solution[58], 25.7 ml/l stock solution A; 25.7 ml/l stock solution B and 2.4 ml/l 4 M KOH (where stock solution A contained the following: KCl 28.80 g/l; KH_2PO_4 60.8 g/l; $NaNO_3$ 240 g/l, at pH 5.5 (adjusted using KOH) and stock solution B contained: MgSO4·7H2O at 20.80 g/l). Selection for the terbinafine marker based *macR*:OE cassette and all CRISPRa vector carrying transformants was carried out using 1.1 µg/ml terbinafine hydrochloride (Sigma-Aldrich) in the solid transformation medium. Terbinafine was supplemented in all media of consecutive experiments, whereas selection for *penDE*-CP_*DsRed* and P*pcbC*-*ble*-t*CYC*1 co-transformation was done using medium containing 50 µg/ml phleomycin (Invivogen, San Diego, CA). For each strain, 2 separate transformant colonies were selected as replicates and re-streaked individually on solid R-agar (see below) medium and cultivated for 7 days on 25°C to produce spores, which were immobilized on lyophilized rice grains and used for further experiments. Schematic representation of engineering DS68530_*penDE*-CP_*DsRed* and *macR*:OE control strains, using CRISPR/Cas9 mediated homologous recombination-based co-transformation into DS68530, is shown on Supp. Figure S1. For each created strain, transformed DNA is listed in Supp. Table S6.

For shake-flask liquid cultures, spores immobilized on lyophilized rice grains ($0.2x10^6$ - $2x10^6$ spores/ml) were precultured for 24 hours in YGG medium[59] before inoculation (1:7.5) into 30 ml Secondary Metabolite Producing (SMP) medium[59] (Penicillin Producing Medium -PPM- without supplemented phenoxyacetic acid or phenylacetic acid), supplemented with 1.1 µg/ml terbinafine. Cultures were grown at 25°C in a rotary incubator at 200 RPM for 5 days, after which mycelium was collected for total RNA extraction as well as extraction of secondary metabolites by vacuum filtration over filter paper. Solid R-agar medium[58] was used for sporulation, SMP-agar (SMP medium supplemented with 15 g/l agar-agar) was used for cultivation, and for secondary metabolite extraction. All solid agar cultures were incubated at 25°C.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from mycelium collected from cultures grown in SMP for 5 days at 25°C. Wet biomass (~200 mg) was added to a screw cap tube containing 900 µl Trizol reagent (Thermo Fisher Scientific, Waltham, MA), 125 µl chloroform and glass beads (ø 0.75-1 mm, 500-600mg). The samples were stored at -80°C until RNA isolation. The mycelium was disrupted using the FastPrep FP120 system (Qbiogene, Carlsbad, CA), followed by total RNA isolation using the phenol-chloroform extraction method. In short, after cell disruption phases were separated by centrifugation (10 min at 14000 x g, the upper phase was transferred to a new tube, followed by a chloroform extraction step, phase separation: 5 min at 12000 x g). RNA was precipitated by the addition of 1 volume isopropanol and incubated on ice for at least 10 minutes, followed by centrifugation (10 min at 12000 x g). Finally, the RNA was resuspended in milliQ H_2O. DNAse treatment was done using the TURBO DNA-free Kit (Thermo Fisher Scientific, Waltham, MA), and the RNA concentration was determined using Nanodrop. cDNA was synthesized using RevertAid H Minus First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions for highly structured mRNAs using oligo $(dT)_{18}$ primers and 1µg of total RNA as template in a 20 µl reaction.

qPCR analysis

Primers used to analyze expression *DsRed*, *macR*, *macA* and m*acJ* can be found in Supp. Table S7. Primers were, when possible, designed to overlap an intron-exon junction to avoid amplification on gDNA. The γ-actin gene (*Pc20g11630*) was used as a control for normalization. The 25 µl qPCR reaction contained 4 µl of a 20x diluted cDNA synthesis reaction, 0.6 µM each of forward and reverse primer, and 12.5 µl SensiMix SYBR Hi-ROX master mix (Meridian Bioscience, Memphis, TN). Expression levels were determined with a MiniOpticon system (Bio-Rad, Hercules, CA) using the Bio-Rad CFX manager software, the threshold cycle (*Ct*) values were determined automatically by regression. Thermocycler conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. Thereafter, a melting curve was generated to determine the specificity of the qPCRs.

LC-MS sample preparation

For secondary metabolite analysis samples were taken from vacuum filtered mycelium or from solid SMP-agar medium as 3x1 cm diameter plugs. The plugs were transferred to a 4.0 ml glass vial and 1 ml acetone supplemented with 4 µl n-Dodecyl-β-D-maltoside (DDM) (10 mg/ml in methanol) was added as internal standard. The plugs were extracted ultrasonically for 60 min, after which the extracts were transferred to a clean vial and dried under a nitrogen stream at 25°C. Dried extracts were resuspended in 200 µl methanol:milliQ-H_2O (1:1) and filtered via a 0.2 µm PTFE syringe filter before the used for LC-MS analysis.

LC-MS metabolite analysis

Metabolite analysis was performed using an Accella1250 UHPLC system coupled to a benchtop ESI-MS Orbitrap Exactive (Thermo Fisher Scientific, Waltham, MA) mass-spectrometer. A sample of 5 µl was injected onto a Waters Acquity CSH C18 UPLC (UHPLC) column (150x2.1 mm, 1.7 µm particle size) operating at 40°C with a flow rate of 300 µl/min. Separation of the compounds was achieved by using a water-acetonitrile gradient system starting from 90% of solvent A (milliQ-water)

and 5% solvent B (100% acetonitrile). 5% of solvent C (2% formic acid) was continuously added to maintain a final concentration of 0.1% of formic acid in the mobile phase. After 5 minutes of initial isocratic flow, the first linear gradient reached 60% of B at 30 minutes, and the second 95% of B at 35 minutes. A purge step for 10 minutes at 90% of B was followed by column equilibration for 15 minutes at the initial conditions. The column eluent was directed to a HESI-II ion source attached to the Exactive Orbitrap mass spectrometer operating at the scan range (m/z 80 – 1600 Da) and alternating between positive/negative polarity modes for each scan. LC-MS data were analyzed using the Thermo Scientific Xcalibur 2.2 processing software by applying the Genesis algorithm for peak detection and manual integration on the sum of the whole spectra of selected ions. The extracted ion counts of investigated compounds were normalized to the DDM internal standard and represented relative to the average detected values from the *MacR*:OE strain replicates. In addition to LC-MS only UV-VIS absorption was monitored at 220, 354 and 700 nm. Ions corresponding to the $[M+H]^+$ pseudo molecular ions of the final steps of the macrophorin biosynthesis pathway (macrophorin A, macrophorin D and 4'-oxomacrophorin D) were identified in chromatographic peaks (1), (2) and (3) respectively and were selected for further analysis. The peaks recorded by each channel for (1), (2) and (3) in match in retention time. The chromatogram recorded at 700 nm showed the best signal-to-noise ratio. (Fig. 3f, Supp. Fig. S4). Due to the necessity of adding an in-line UV-VIS detector between the MS and the column to generate UV-VIS chromatograms, small discrepancy in Rt between different datasets was observed.

Biolector

Spores (immobilized on 20 rice grains) were used to inoculate 10 ml SMP and cultures were incubated for 48 h in a rotary incubator at 200 rpm and 25°C. For BioLector analysis and analysis of growth in FlowerPlate (MTP-48-B) wells, this pre-grown mycelium was diluted 8 times in fresh SMP, supplemented with 1.1 µg/ml terbinafine (except for parent strain DS68530). The 1 ml cultures were

grown in the BioLector micro-bioreactor system (M2Plabs, Baesweiler, Germany), shaking at 800 rpm at 25°C. In the BioLector, biomass was measured via scattered light at 620 nm excitation without an emission filter. The fluorescence of *DsRed-SKL* was measured every 30 min with "DsRed I" 550 nm (bandpass: 10 nm) excitation filter and 580 nm (bandpass: 10 nm) emission filter. Data were obtained from 3 separate experiments, each consisting of 2-3 biological replicates. The data obtained from the BioLector experiments were analyzed and presented using the TIBCO Spotfire Software (TIBCO Software Inc., Palo Alto).

Fluorescence microscopy

For visualization of DsRed-SKL fluorescent protein, liquid shake-flask cultures were cultivated for 5 days in SMP, and mycelium was collected and re-suspended in phosphate-buffered saline (58 mM Na_2HPO_4; 17 mM NaH_2PO_4; 68 mM NaCl, pH 7.3). Confocal imaging was performed on a Carl Zeiss LSM800 confocal microscope using 20x objective and ZEN 2009 software (Carl Zeiss, Oberkochen, Germany). The DsRed signal was visualized by excitation with a 543 nm helium neon laser (Lasos Lasertechnik, Jena, Germany), and emission was detected using a 565 to 615 nm band-pass emission filter[60].

Bio-assay

Macrophorin producing strains were tested for antimicrobial activity against *Micrococcus luteus* as follows: Supernatant of *P. rubens* strains carrying either the pAMA18.0, pAMA18.4, pAMA18.5 vector and the *macR*:OE strain was collected after 5 days of growth in liquid SMP medium and concentrated 10x in an Eppendorf Concentrator Plus (30°C, vacuum for aqua solutions setting). An overlay of soft LA-agar (1%) inoculated with *M. luteus* to an OD600 of 0.125 was poured on top of an agar (1%) bottom layer with Oxford Towers (8x10 mm) spaced out evenly. The Oxford Towers were removed aseptically and 100 µl of the 10x concentrated supernatant was loaded in the resulting wells as indicated. The plate was incubated at 30°C for 24 hours before imaging. The experiment was performed in triplicate.

Abbreviations

BGC, Biosynthetic gene cluster; CRISPRa, CRISPR activation; SM, Secondary metabolite; cDNA, complementary DNA; sgRNA, chimeric single guide RNA; RNP, ribonucleoprotein complex; NLS, nuclear localization signal; CP, Core promoter; TSS, Transcription start site

Author contributions

L.M. and M.H. contributed equally to this work. LC-MS analysis was carried out by N.A.W.K and L.M. BioLector analysis was carried out by Y.N.. L.M. designed the experiments. L.M. and M.H. carried out all other experiments and wrote the manuscript with critical feedback and help from N.A.W.K., R.A.L.B., Y.N., and A.J.M.D.. Y.N., R.A.L.B., and A.J.M.D. conceived the original idea.

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Supplementary information

Table S1: CRISPR and CRISPRa sgRNA targeting sequences with corresponding CRISPRater scores.

Name	sgRNA targeting sequence (5'-3')	CRISPRater score (CCtop)
PenDE-sgRNA_a	GACCATAGCATGACACTGAT	0.54
PenDE-sgRNA_b	GTCCCATCAGTGTCATGCTA	0.60
PenDE-sgRNA_c	ATTGGCCGTAGCCACCAATC	0.76
PenDE-sgRNA_d	ATGCTATGGTCCCAGATTGG	0.81
PenDE-sgRNA_e	CACTGATGGGACATCAACTG	0.28
PenDE-sgRNA_f	GGATGCAGCAGGGATACTCG	0.40
MacR-sgRNA_1	AAAAACCCACCTCCCCTCAG	0.73
MacR-sgRNA_2	ACTCCTCCTCTGAGGGGAGG	0.69
MacR-sgRNA_3	CTGCATGCGAACTCCCAATA	0.60
MacR-sgRNA_4	GGAGTTCGCATGCAGAGAAG	0.84
MacR-sgRNA_5	CTCGCCCGGGAGGTAATTGG	0.73
MacR-sgRNA_6	TTAGCCCCCAATTACCTCCC	0.71
MacR-sgRNA_7	CAACTTCTCAAACATCGTTC	0.78
MacR-sgRNA_8	GTTTGAGAAGTTGGTGTTGG	0.70
MacR-sgRNA_9	GCTATAACACGAAGATGACA	0.52
MacR-sgRNA_10	TAATAGGCAGGAAAGATTCG	0.77
MacR-sgRNA_11	TATGTTTTGAAGTATACCCG	0.84
MacR-sgRNA_12	ACATCTGTAGTGCTTACCTC	0.68
MacR-sgRNA_13	GGATTTTTCACGATACGGGG	0.66
MacR-sgRNA_14	CCCCGTATCGTGAAAAATCC	0.67
MacR-sgRNA_15	TATAAACCTCACAGATCTCT	0.48
MacR-sgRNA_16	GCAGAGCCAAGAGATCTGTG	0.79
MacR-sgRNA_17	TGTCCTCCCTTAGCAGTTTG	0.73
MacR-sgRNA_18	AAGCCCCAAACTGCTAAGGG	0.69
MacR-sgRNA_19	TCCAGACGCCATCCGTTTCG	0.76
MacR-sgRNA_20	ACCACGAAACGGATGGCGTC	0.61
T7-pen-loci-editing[14]	GAACCAACATCATTAAGCAG	0.70
T7-*macR*:OE-editing[14]	AATGTTCCACTCCTCCTCTG	0.85

Table S2: Macrophorin related masses identified by LC-MS in *macR*:OE from SMP-agar plug extracts after 10 days of growth.

Nr	Compound	Formula	Theoretical mass m/z $[M+H]^+$	Theoretical mass m/z $[M-H]^-$	t_{R*} (+ mode)	t_{R*} (- mode)	Selected Rt for mass measurement	Detected mass m/z $[M+H]^+$	Detected mass m/z $[M-H]^-$	PPM error (+ mode)	PPM error (- mode)
1	macrophorin A	$C_{22}H_{32}O4$	361.23734	359.22276	33.15	33.15	33.15	361.23776	359.22200	**1.16**	**-2.12**
2	macrophorin D	$C_{28}H_{40}O_8$	505.27959	503.26502	33.91	33.91	33.91	505.27999	503.26441	**0.79**	**-1.21**
3	4-oxomacro-phorin D	$C_{28}H_{38}O_8$	503.26394	501.24937	27.62/ 28.08/ 28.46/ 35.92	35.92	35.92	503.26469	501.24971	**1.49**	**0.68**
4	DDM	$C_{24}H_{46}O_{11}$	511.31132	509.29672	28.12	28.12	28.12	511.3128	509.29678	**2.89**	**0.12**

Table S3: LC-MS metabolite analysis of macrophorin related metabolites from fungal SMP-agar plug extracts after 10 days of growth. Data represented as ion intensity normalized to added internal standard DDM.

Strain ID	macrophorin A	macrophorin D	4'-oxomacrophorin D
AMA18_0	0	0	1,436
AMA18_1	0	0	12,470
AMA18_2	0	0	4,473
AMA18_3	0	0	5,843
AMA18_4	117,925	117,925	80,451
AMA18_5	771,392	77,170	52,530
AMA18_6	2,646	0	1,989
AMA18_7	3,456	0	418
AMA18_8	0	827	1,157
AMA18_9	1,391	449	0
AMA18_10	0	0	3,899
AMA18_11	800	0	1,947
AMA18_12	0	0	2,857
AMA18_13	0	0	5,682
AMA18_14	0	636	1,071
AMA18_15	0	811	3,323
AMA18_16	0	0	5,503
AMA18_17	0	764	1,491
AMA18_18	956	0	806
AMA18_19	0	1,321	0
AMA18_20	0	1,691	3,113
***MacR*:OE**	3,675,166	2,747,993	91,620

Table S4: Oligonucleotide primers used for strain and vector construction.

Part ID	Description	Template	Primer Pair Sequences (5’->3’)
A	*penDE*-CP_*DsRed-T1-SKL*-*Tact* (80-800 bp flanks)	pVE2_10[36] AddGene ID #154228	F:TCGACACGCTTTACGAATTCCCATGG R:GATATGCCGTCTGCAGAGACTGCGATA
B	P*pcbC*-*ble*-T*cyc1* (80bp flanks)	pJAK-109[55]	F:AGACTCGGTGATGCAGCAAATAGCGACTGTTCGTTGCGGGGTC CGAACCCGCTCGGCAGCACCGGGCTCTCCCTACTATCCCTCGA TAGCAGTCGACTACATGTATCTGC ATGTTGCATC R:TGACACTGATGGGACATCAACTGGGGCACCTCGAGTATCCCTGCTGCATC CGCCTAGTCTCTCCCCATGGGAATTCGTAAAGCGTGTCGATAA GCTTGCAAATTAAAGCCTTCGAGCG
C	P*pcbC*-*macR* (80bp flanks)	pVE2_19[36] AddGene ID #154241	F:GCTGCATTGGTCTGCCATTGC R:CGAACCTTGCGTCGTCGGCAGACGATGCAACTGAGCGATAACCG GGGTTTCTTGATTTGGCGGGGCTCGGATAAAGGCATTGGTGTCTAGA AAAATAATGGTGAAAACTTG
D	*PgpdA*-*ergA*-T*amdS* terbinafine marker (80bp flanks)	pCP1_45[41]	F:CGCCCTGCTATCCCAACCCTGCACTTGTCCTCTTCTCTGCATGCG AACTCCCAATATGGTCACGATAAAAACCCACCTCCTACCGCTC GTACCATGGGTTGAG R:CAGTGCTTCAGTCGCCCAGATTCTCGATGGAGATTGGCCAGGTCAGCC ATATATACCCTGCAATGGCAGACCAATGCAGCGAATTCGAGCTCGGAGTGG ATCC
E1	Cas9m4-VPR-1	pYTK036[54] AddGene ID #65143	F:TGAAGACTTAATGGACAAGAAGTATTCTATCGGACTGGCCATCGGGACT AATAG R:TGAAGACTTGCAGCCACGTCGTAGTCTGAGAGC
E2	Cas9m4- VPR-2	Annealed oligos	F:TGAAGACTTCTGCCATCGTCCCTCAGAGCTTCCTCAAAGACGACTCAA TTGACAATAAGGTGCTGACTCGCTCAGACAAGGCCAAGTCTTCA R:TGAAGACTTGGCCTTGTCTGAGCGAGTCAGCACCTTATTGTCAATTGAG TCGTCTTTGAGGAAGCTCTGAGGGACGATGGCAGAAGTCTTCA
E3	Cas9m4- VPR-3	pYTK036[54] AddGene ID #65143	F:TGAAGACTTGGCCCGGGGAAAGTCAGATAACGTGC R:TGAAGACTTATCCCCTCCGAGCTGTGAGAGG
E4	Cas9m4- VPR-4	pAG414GPD-dCas9-VPR[16] AddGene ID #63801	F:GCTGAAGACTTGGATAGCAGGGCTGACCCCAAGAAGAA R:TGAAGACTAAAGCTCAAAACAGAGATGTGTCGAAGATGGACAGT
F1	HH-sgRNA-HDV “plug-and-play” 1	pFC334[33] AddGene ID #87846	F:CGGTCTCTAGCGGCGTAAGCTCCCTAATTGGCCC R:CGGTCTCATCGGTGATGTCTGCTCAAGCGG
F2	HH-sgRNA-HDV “plug-and-play” 2	pICH41308[53] AddGene	F:GAAGACTCCCGACGAGACCCAGCTGGCACGACAGGTTTC R:GAAGACAAAAACGGAGACCACAGCTTGTCTGTAAGCGGATG
F3	HH-sgRNA-HDV “plug-and-play” 3	pFC334[33] AddGene ID #87846	F:TGGTCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG R:CGGTCTCAGGAGGAGCCAAGAGCGGATTCCTCAGTCTCGTACGTCTC
G1	Gibson unit 1, dCas9m4-VPR	pLM1_135	F:GAATTCCTGCAGCCCCAGATCATCCTGTCTTCAGTCTTAACGCTGCAA GAATTCAAGCTTGGAG R:TGGGATGTTCCATGGTAGCTGTGAA
G2	Gibson unit 2, P*40S* flank with *PgpdA*-*ergA*-T*amdS*	pCP1_135	F:CAAGGTTCTTCTCGAAGTAGTTGTTCT R:CGCTCGTACCATGGGTTGAG
G3	Gibson unit 3, sgRNA “plug-and-play”	pLM1_135	F:ACAGGTGACTCTGGATGGC R:ACCTTCAATATCAACTCTTTCAGGGGGGGAGCGGCCT TAAGTCGGCAACGAGAGGTATGTCTAAAGT
H	T7-sgRNA-transcription template[14] (penicillin-loci)	overlapping oligonucleotides	F:ATGTAATACGACTCACTATA**gAACCAACATCATTAAGCAG** GTTTCAGAGCTATGCTGGAAA* R:AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAAC GAACTAGTCTTATTTCAACTTGCTATGCTGTTTCCAGCATAGCTCTGAAAC
I	T7-sgRNA-transcription template[14] *macR*-OE	overlapping oligonucleotides	F:ATGTAATACGACTCACTATAg**AATGTTCCACTCCTCCTCTG**GTTTC AGAGCTATGCTGGAAA* R:AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGA ACTAGTCTTATTTCAACTTGCTATGCTGTTTCCAGCATAGCTCTGAAAC

*: 20bp sgRNA target sequence shown in bold, lowercase “g” indicates T7 transcription site

Table S5: Modular Cloning and Gibson Assembly based vector construction.

Created vector	Description	Cloned Part IDs or MoClo units	Recipient vector
LM0_36	dCas9m4-2xNLS-VPR	E1, E2, E3, E4	pICH41308[53]
LM1_100	P*40S*-dCas9m4-2xNLS-VPR-T*tif35*	pZB0_21[36], pLM0_36, pYN0_10[36]	pICH47742[53]
LM1_113	sgRNA "plug-and-play" transcription unit (P*gdpA*-lacZ-HDV-T*trpC*)	F1, F2, F3	pICH47761[53]
LM2_135	P*40S*-dCas9m4-2xNLS-VPR-T*tif35*, P*gpdA*-*ergA*, sgRNA "plug-and-play" transcription unit, PenFlanks, MoClo End-Linker on LVL2 MoClo backbone vector	pZB1_1[36], pLM1_100, pCP1_45[41], pLM1_113, pZB1_2[36], pICH41800[53]	pICH50505 (alternative of pAGM4673[53])
pAMA18.0*	P*40S*-dCas9m4-2xNLS-VPR-T*tif35*, P*gpdA*-*ergA*-T*amdS*, sgRNA "plug-and-play" transcription unit on AMA1 backbone vector	G1, G2, G3	pJAK-109 based linearized AMA1 vector[55]

*Vector constructed using Gibson Assembly

Table S6: Fungal strains created in this study.

Strain ID	Transformed DNA	Transformed strain	Transformation method
penDE-CP_ *DsRed*	Part ID A and B	DS68530	PEG mediated RNP-based CRISPR/Cas9 editing by homologous recombination[13,14] sgRNA transcribed using T7 polymerase from DNA of "Part ID: H"
macR:OE	Part ID C and D	DS68530	PEG mediated RNP-based CRISPR/Cas9 editing by homologous recombination sgRNA transcribed using T7 polymerase from DNA of "Part ID: I"
AMA18.0_*DsRed* (no-sgRNA control for *DsRed*)	pAMA18.0	DS68530_*penDE*-CP_*DsRed*	PEG mediated vector transformation
AMA18.a	pAMA18.a	DS68530_*penDE*-CP_*DsRed*	PEG mediated vector transformation
AMA18.b	pAMA18.b	DS68530_*penDE*-CP_*DsRed*	PEG mediated vector transformation
AMA18.c	pAMA18.c	DS68530_*penDE*-CP_*DsRed*	PEG mediated vector transformation
AMA18.d	pAMA18.d	DS68530_*penDE*-CP_*DsRed*	PEG mediated vector transformation
AMA18.e	pAMA18.e	DS68530_*penDE*-CP_*DsRed*	PEG mediated vector transformation
AMA18.f	pAMA18.f	DS68530_*penDE*-CP_*DsRed*	PEG mediated vector transformation
AMA18.0 (no-sgRNA control for *macR*)	pAMA18.0	DS68530	PEG mediated vector transformation
AMA18.1	pAMA18.1	DS68530	PEG mediated vector transformation
AMA18.2	pAMA18.2	DS68530	PEG mediated vector transformation
AMA18.3	pAMA18.3	DS68530	PEG mediated vector transformation
AMA18.4	pAMA18.4	DS68530	PEG mediated vector transformation
AMA18.5	pAMA18.5	DS68530	PEG mediated vector transformation
AMA18.6	pAMA18.6	DS68530	PEG mediated vector transformation
AMA18.7	pAMA18.7	DS68530	PEG mediated vector transformation
AMA18.8	pAMA18.8	DS68530	PEG mediated vector transformation
AMA18.9	pAMA18.9	DS68530	PEG mediated vector transformation
AMA18.10	pAMA18.10	DS68530	PEG mediated vector transformation
AMA18.11	pAMA18.11	DS68530	PEG mediated vector transformation
AMA18.12	pAMA18.12	DS68530	PEG mediated vector transformation
AMA18.13	pAMA18.13	DS68530	PEG mediated vector transformation
AMA18.14	pAMA18.14	DS68530	PEG mediated vector transformation

Strain ID	Transformed DNA	Transformed strain	Transformation method
AMA18.15	pAMA18.15	DS68530	PEG mediated vector transformation
AMA18.16	pAMA18.16	DS68530	PEG mediated vector transformation
AMA18.17	pAMA18.17	DS68530	PEG mediated vector transformation
AMA18.18	pAMA18.18	DS68530	PEG mediated vector transformation
AMA18.19	pAMA18.19	DS68530	PEG mediated vector transformation
AMA18.20	pAMA18.20	DS68530	PEG mediated vector transformation

Table S7: Oligonucleotide primers used for qPCR analysis.

Gene	Sequences (5'->3')
DsRed-T1-SKL	F:CCAAGGTGTACGTGAAGCAC R:CCTTGTAGATGAAGGAGCCGT
macR (*Pc16g00410*)	F:GACGACGCAAGGTTCGCT R:GTCCTGCGGTGATACTGGTC
macA (*Pc16g00370*)	F:CGGGTTCAAACACGTCCGTA R:CATCCAGTGCAACGCTAGGA
macJ (*Pc16g00320*)	F:TCTTGGGGAATTTGGTGGACA R:CAGACCCGATACTACCCAGC
γ-actin (*Pc20g11630*)	F:CTGGCGGTATCCACGTCACC R:AGGCCAGAATGGATCCACCG

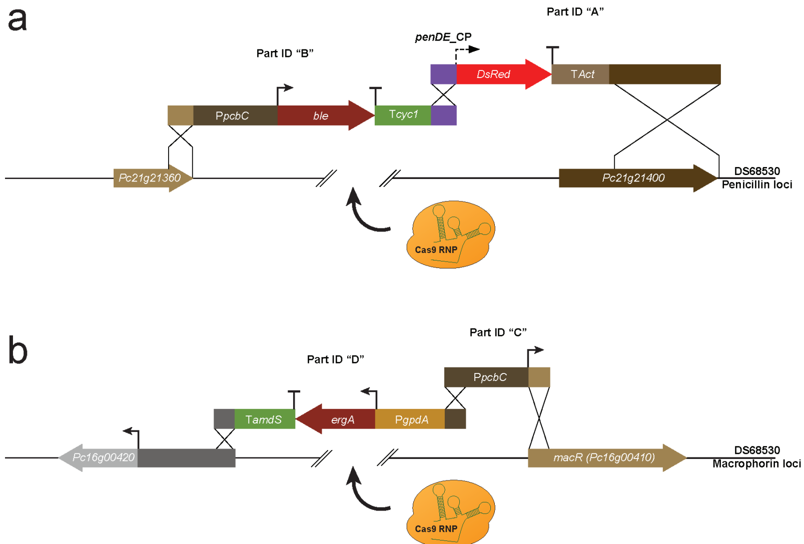


Figure S1: Schematic representation of CRISPR/Cas9 mediated co-transformation into DS68530 and engineering of *penDE*-CP_*DsRed* **(a)** and *macR*:OE **(b)** fungal strains, using Part ID A,B and C,D respectively. The *ergA* (terbinafine) or *ble* (phleomycin) marker provide selection and flanking regions for recombination with the marker-free cassette and the genomic DNA.

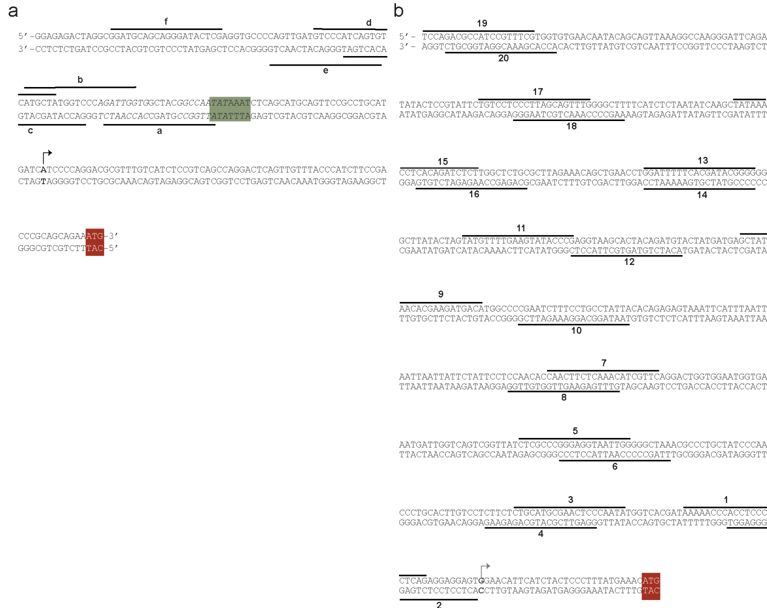
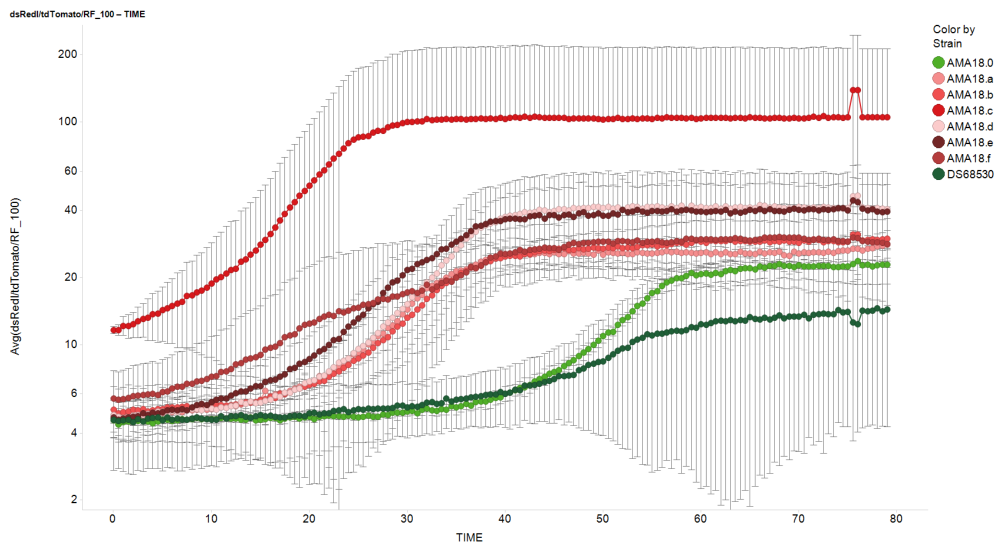
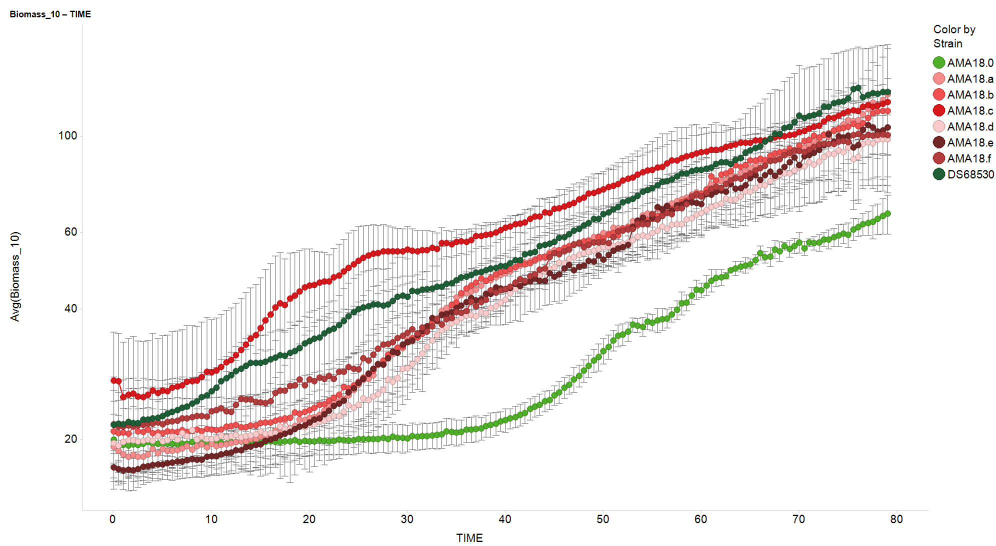


Figure S2: Representation of sgRNA targeting sequences on promoter sequences of *penDE*-CP (a) and *macR* (b). Transcription start site (TSS) of *penDE* indicated as black arrow, predicted TSS of *macR* indicated as gray arrow. Red boxes indicate translation start codon. The green box indicates the TATA-box.

a)



b)



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c)

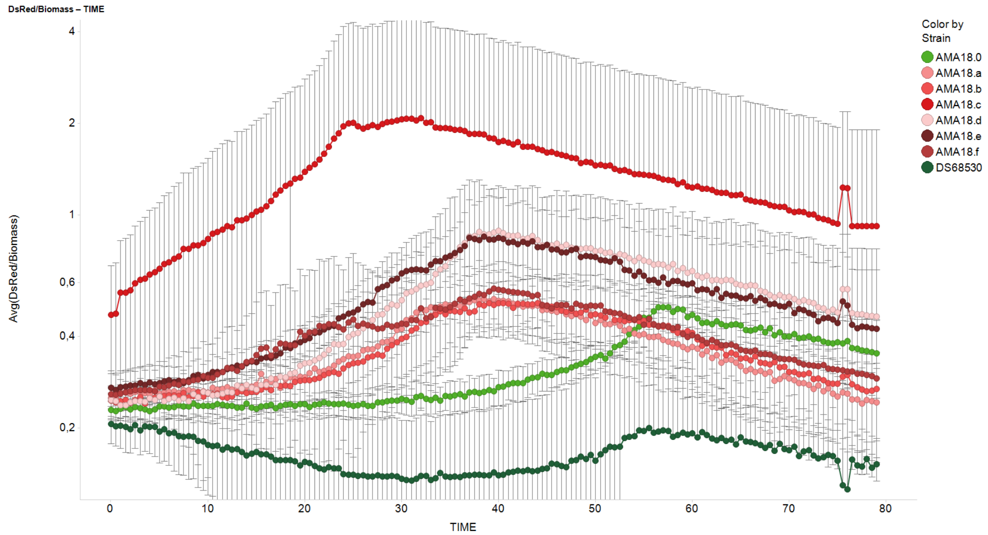

Figure S3: Development of **(a)** biomass **(b)** DsRed fluorescence **(c)** and DsRed fluorescence corrected for biomass for indicated CRISPRa strains in BioLector microbioreactor, compared to non-sgRNA control (AMA18.0) and *DsRed*-free parent strain (DS68530). Strains were cultivated in SMP liquid media, supplemented with terbinafine (except parent strain DS68530). Data were obtained from 3 separate experiments, each consisting of 2-3 biological replicates; error bars show the standard deviation.

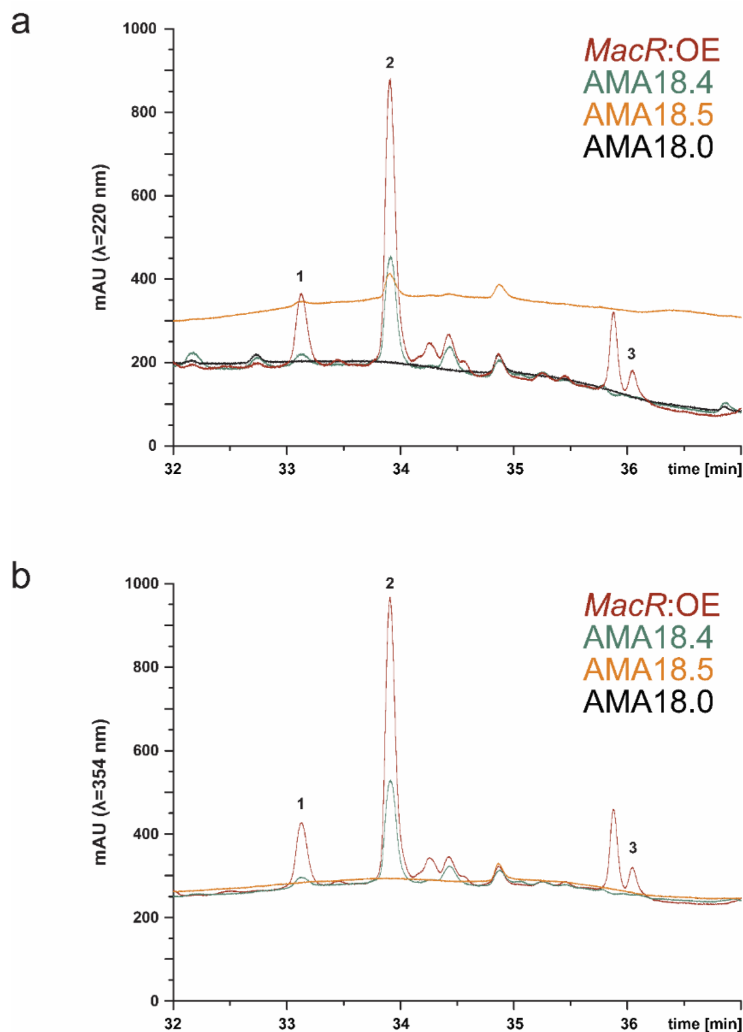

Figure S4: LC-MS UV-VIS chromatograms of hyphae extracts of CRISPRa and *macR*:OE strains analyzed at (**a**) λ=220 nm and (**b**) λ=354 nm.

Note S1 Complementary DNA sequence of macR (Pc16g00410) of *Penicillium rubens* DS68530 as determined by Sanger sequencing (Accession ID: MZ310414.1).

Note S2 DNA sequence of dCas9-VPR fusion and description of its domains on vector pLM-AMA18.0-dCas9-VPR.

Note S1 and Note S2 are available in the online version of this manuscript at:
https://www.nature.com/articles/s41598-020-80864-3

CHAPTER 5

Modular Synthetic Biology Toolkit for Filamentous Fungi

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Abstract

Filamentous fungi are highly productive cell factories, often used in industry for the production of enzymes and small bioactive compounds. Recent years have seen an increasing number of synthetic biology-based applications in fungi, emphasizing the need for a synthetic biology toolkit for these organisms. Here, we present a collection of 96 genetic parts, characterized in *Penicillium* or *Aspergillus* species, that are compatible and interchangeable with the Modular Cloning system. The toolkit contains natural and synthetic promoters (constitutive and inducible), terminators, fluorescent reporters, and selection markers. Furthermore, there are regulatory and DNA-binding-domains of transcriptional regulators, and components for implementing different CRISPR-based technologies. Genetic parts can be assembled into complex multipartite assemblies and delivered through genomic integration or expressed from an AMA1-sequence-based, fungal-replicating shuttle vector. With this toolkit, synthetic transcription units with established promoters, fusion proteins or synthetic transcriptional regulation devices can be more rapidly assembled in a standardized and modular manner for novel fungal cell factories.

Keywords: Synthetic biology toolkit, Modular Cloning, Hybrid transcription factor, Inducible promoter, Transcriptional regulation, Filamentous fungi

Introduction

Filamentous fungi are widely used as cell factories: organic acids, small molecule drugs, homologous as well as heterologous proteins expressed in fungi are applied in various industries, and fungal biotechnology is considered as an innovation driver for a circular economy.[1] Fungi are not only excellent workhorses for protein production due to their natural capacity for protein secretion, but fungal genomes also contain a large number of biosynthetic gene clusters (BGCs), encoding potentially useful natural products. The core enzymes of these natural product producing clusters are usually nonribosomal peptide synthetases (NRPSs) polyketide synthases (PKSs), or terpene synthases (TPSs). Advanced bioinformatics tools predict about 30-70 BGC per fungal species.[2] It has become obvious that next to known natural products, fungal genomes hold an enormous amount of untapped biosynthetic potential in the form of transcriptionally silent, uncharacterized BGCs.[2] These "cryptic" BGCs, which are usually not expressed in laboratory conditions, can potentially provide new leads for novel natural products. Single species like *Aspergillus nidulans* or *Penicillium rubens* contain over 30 NRPSs and PKSs, responsible for natural product biosynthesis, of which most are still awaiting characterization.[3,4]

Synthetic biology has revolutionized metabolic engineering by providing new tools to create modular, synthetic genetic circuits for controlled activation and/or fine-tuning expression of specific genes or complete BGCs, thereby optimizing the production of endogenous or exogenous proteins and secondary metabolites.[5–11] In addition to "rewiring" pathways which are already transcriptionally active, such tools can be used for the activation of transcriptionally silent BGCs, and novel natural product discovery. Synthetic genetic circuits provide a new way of transcriptional regulation by mimicking natural regulatory mechanisms. Synthetic transcription factors (STFs) can be employed to achieve transcriptional regulation, and in their simplest design, are fusions between the DNA-binding domain (DBD) of a known transcription factor and a transcriptional regulator (activator or repressor). As the DBD of a TF binds to its specific upstream activating sequence (UAS) in the targeted promoter, the

strength of the regulation can be increased by integrating additional UASs in a synthetic promoter. These systems are further tunable by utilizing inducible promoters to titrate the protein levels of the corresponding transcription factors or other genetic switches. Using such synthetic transcriptional regulators, gene activation or repression can be achieved in a controlled manner, or transcription can be fine-tuned for each gene individually.[5–7] Synthetic expression systems have previously been demonstrated in *Aspergilli*[5,6,8,10], *Trichoderma reesei*[9], *P. rubens*[7], and *Ustilago maydis*[11]. For instance, the bacterial doxycycline/ tetracycline-inducible system has been adopted for *Aspergillus* and *U. maydis* providing an inducer-based transcriptional regulation.[5,8,11] STF-based regulatory systems show transferability between a variety of different fungi.[6,12] Next to methods which require introducing genetic parts permanently into the host organism genome, plasmid-based alternatives are also available for filamentous fungi, as well as CRISPR-based technologies for transcriptional regulation[13–15]. All these synthetic biology-based tools provide new alternatives to further aid the exploitation of fungal workhorses.

Targeted DNA delivery and precise genome editing is often required for constructing STF-regulated genetic circuits. Engineering of non-domesticated strains is often time-consuming and engineering efforts show low efficiency. Targeting efficiency of the integrated donor-DNA to the designated loci can be increased by using long homologous fragments of genomic DNA of the host organism. More accurate genome editing is possible with strains devoid of the fungal homologues of the *ku70* or *ku80* genes, as homology-directed repair (HDR) will be favored over the non-homologous end-joining (NHEJ) DNA repair pathway.[16] In some (non-domesticated) fungal isolates genome engineering can be less efficient due to the presence of the NHEJ machinery, resulting in more random integration events. In such strains, DNA delivery using non-integrative fungal shuttle vectors can be advantageous, as this method does not rely on genomic integrations. The autonomous replication providing AMA1-sequence supports episomal DNA delivery in several species of filamentous fungi and shuttle vectors for containing this sequence are commonly used.[17] Such vectors

enable rapid genetic circuit assembly for gene expression in the fungal host. Fungal shuttle vectors are commonly used to deliver the *in vivo* expressed components of the CRISPR-Cas (CRISPR-associated protein) genome-editing technology in filamentous fungi[18], which further allows for swift and reliable genomic engineering.

Modular toolkits allow rapid construction of genetic circuits, various STFs and protein fusions in a combinatorial manner, through recombining already available or via incorporation of new genetic parts into the established system.[19] Standardized, characterized genetic parts are key elements for rapid and modular construction of novel genetic circuits. In modular cloning systems typically the genetic elements (as PCR products or synthetic DNA) first inserted into entry vectors (level 0) to create genetic parts. These basic genetic parts (also termed as modules) are then used for the next step of the assembly into transcription units (level 1), which can be further combined into genetic circuits containing multiple transcription units (level 2).[19] The Golden Gate Assembly-based Modular Cloning (MoClo) system supports the assembly of several transcription units on one single plasmid, where the number of units is only limited by the host's tolerance for the size of plasmid DNA.[19] A limitation of the Golden Gate Assembly line is the initial cloning step which often requires the removal of type IIS recognition sites used by MoClo, through PCR amplification or DNA synthesis. This initial work can be reduced by using parts made available through repositories for synthetic toolkits, which could contribute to a more rapid assembly of novel synthetic circuits for various organisms. Synthetic modular vector collections (toolkits) are publicly available for bacteria[20], various yeasts[21,22], plants[23], and mammalian host[24] cell lines. Although collections of Golden Gate-based vectors were recently established in *A. niger*[25] (GoldenMOCS) and deposited on Addgene for metabolic pathway construction[25], or in *Sordaria macrospora and P. rubens*[26] for protein fusions and gene deletions, a substantial collection of generic tools for synthetic biology applications in filamentous fungi is not yet deposited and available in global nucleic acid repositories.

Modular assemblies provide high flexibility regarding assembly compared to systems which leave an "assembly scar" after cloning. As the genetic parts in such systems are flanked with Type IIs restriction enzyme cut sites, as the restriction happens outside their recognition sequence, the created cohesive sequences can be used for one-pot "scarless" cloning approaches. These cohesive linker sequences mark the predetermined location for the genetic element in an assembled transcription unit, and are used for the assembly of multiple transcription units as well. For example, in the standard MoClo language[19] a transcription units for cytosolic proteins consists of promoters (P), untranslated regions (U), coding sequences (CDS) and terminators (T), using the GGAG-(P)-TACT-(U)-AATG-(CDS)-GCTT-(T)-CGCT 4bp-long linker sequences to connect to each other and to the receiving backbone. This hierarchical structure provides a platform for an easily automatable, rapid assembly of multigene constructs, on the other hand creating limitations for interchanging building blocks from other modular systems. Numerous modular assemblies aimed to improve the standard MoClo assembly[20,21,24], but by changing the linker sequences for transcription unit assembly and without considering backward compatibility, this creates incompatibility between the different modular assembly systems.

This Fungal Modular Cloning Toolkit consists of 96 genetic parts as MoClo compatible entry vectors, including synthetic and native fungal promoters, terminators, selection markers, various coding sequences (CDSs) for transcriptional activation- and DNA-binding domains, fluorescent reporters, the AMA1 sequence for fungal autonomous replication, as well as CRISPR components such as *Cas9*, *dCas9* sequences and sgRNA (single guide RNA) transcription units for filamentous fungi (Figure 1). This generic modular toolkit which provides the building blocks for rapid construction of complex genetic circuits, should be of great use to the field of fungal synthetic biology and accelerate the discovery of bioactive compounds, as well as optimization of their production.

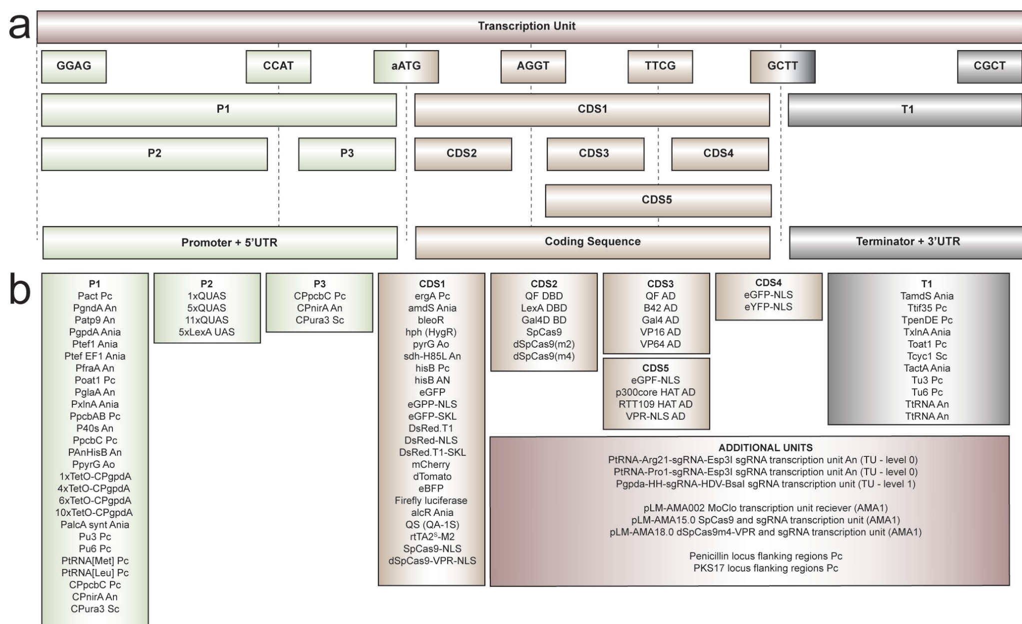

Figure 1. List of vectors in the Fungal Modular Cloning Toolkit. a) Location of genetic parts in a transcription unit with their corresponding linker sequences. b) List of parts of the toolkit containing promoters (P1), UASs (P2), UAS compatible core promoters (P3), coding sequences with various fusion possibilities (CDS1-5), and terminators (T1), complete transcription units (TU) and additional vectors (sgRNA transcription units, flanking sequences, AMA1 vectors). Abbreviations (Pc, An, Ania, Ao, Sc) indicate the origin of the template (*P. rubens*, *A. niger*, *A. nidulans*, *A. oryzae*, *S. cerevisiae*, respectively).

Results and discussion

In this work, we describe a modular synthetic biology toolkit for use in filamentous fungi. Most of the genetic parts in this toolkit originate from *Aspergillus* or *Penicillium* species, or from other established synthetic fungal systems for gene regulation, heterologous expression, and genetic engineering.[5–7,27,28] It is a common observation that promoters and other genomic elements of filamentous fungi are interchangeable between fungal species and are therefore widely used in heterologous filamentous fungal systems.[6,12] The parts of this MoClo toolkit were analyzed in *P. rubens* unless the genetic part was already established or characterized in previous studies as listed in Table 1. All vectors were constructed using the standardized MoClo system, discussed in detail in Weber *et al.*, 2011.[19] This collection of basic genetic parts provides a tool for rapid assembly of various

combinations of parts into multigene genetic circuits, which can be delivered to the host organism through genomic integration or using episomal AMA1 vectors.

Constitutive, inducible, and synthetic promoters

A collection of functional native or synthetic promoters and terminators are essential for a synthetic biology toolkit. The Fungal Modular Cloning Toolkit provides 20 promoters, 3 core promoters, and 11 terminators (Table 1). These genomic elements were previously used in synthetic genetic circuits in *Aspergillus* or *Penicillium* with varying strain background, media and cultivation methods (Table 1).[5–7,28] Others were benchmarked previously in *P. rubens* using fluorescent reporters in a BioLector microbioreactor.[27]

Constitutive promoters deliver stable expression across different growth environments and growth phases. Strong constitutive promoters like the commonly used promoter of *gpdA* (ANIA_08041)[29] from the glycolytic pathway are often used to drive gene expression in *Aspergillus* or *Penicillium.* The *gpdA* promoter is used to constitutively express various genes, as well as fungal selection markers, ribozyme self-cleaved sgRNA or expression of STFs.[5,18] The promoter of the *TEF1* (translation-elongation factor 1a) gene is another common strong and constitutive fungal promoter that has been used for polygalacturonase production and the expression of the SpCas9 encoding gene.[18] The constitutive promoter of the 40S ribosomal protein S8 (An0465, *40S*, *RPS8*) has been shown to give a stable expression of fluorescent reporters, STFs for scalable transcriptional activation[7] and expression of dSpCas9-VPR from *Streptococcus pyogenes* for CRISPR-based transcription activation (CRISPRa).[14] The promoter of *gndA* (An11g02040, 6-phospho-gluconate dehydrogenase) was shown to give an intermediate strength of transcription[27], and proven to be weaker compared to the constitutive An0465 promoter in *P. rubens*.[7] The well-studied promoters of the bidirectional penicillin biosynthesis genes, *pcbAB* (Pc21g21390) and *pcbC* (Pc21g21380) are commonly used as strong promoters. Although *pcbAB* and *pcbC* are under the control of regulation by both nutritional and developmental factors, they provide a strong transcription rate in lactose-based cultivations.[27]

In our toolkit, the constitutive promoter of *oliC31* (An04g08190, mitochondrial ATP synthase subunit 9) is also included, which was shown to provide expression comparable to the promoter of *pcbAB* in *Penicillium*[27] as well as the constitutive promoter of the housekeeping γ-actin (Pc20g11630) from *P. rubens*. Besides reliable and constitutive promoters, stimulus-responsive feedback-loops may require the expression of the regulators at certain time points of the cultivation. Therefore, a set of inducible promoters (P*XlnA* by xylose; P*OAT1* by amino acids; P*glaA* by maltose; P*Tet* by tetracycline; P*alcA* by aldehydes) are incorporated.

An increasing number of promoter libraries have been designed for yeast and filamentous fungi, by creating synthetic promoters for STFs through the combination of various upstream activating sequence (UAS) elements and different core (or minimal) promoters (CP).[6,7] Transcription factor-based specific activation/repression mechanisms interact with the designated UAS elements, but a CP sequence is required to recruit general transcription factors and the RNA polymerase II for transcription initiation.[30] As part of this toolkit, a collection of CPs are included (CP*pcbC*, from *P. rubens*, CP*NirA from A. nidulans*, and CP*URA3* from *S. cerevisiae*), which, in combination with an UASs compatible with a DBD of a STF (1x, 5x, or 11x QUAS for QA-1f-DBD, 5x LexA UAS for LexA-DBD), can create synthetic promoters with expression levels ranging from hardly detectable to a level similar to that of highest expressed native genes.[7] Moreover, entry vectors are provided for the construction of a bacterial-originated tetracycline-inducible (Tet-On) synthetic genetic circuits, including the rtTA2^{S}-M2 (modified TetR-3xVP16) STF and its synthetic promoters using 1, 4, 6 or 10 repeats of TetO UASs.[5]

Various synthetic transcription factors (STFs) (transcriptional activators or repressors) can be constructed using transcription factor domain fusions, where a selected regulator domain can be recruited to a promoter region of the gene of interest.[5–7] These STFs often consist of direct fusion of a DBD and an activation domain (AD). Using the ability of the DBD of a transcription factor to bind to its UAS, these STF fusion proteins can be used to design synthetic transcriptional regulators or genetic control circuits. Viral ADs are widely used to create potent

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STFs, most commonly the VP16 or its tandem repeats (VP64, VP160) from herpes simplex virus. Numerous DBDs of transcription factors have shown to be functional in filamentous fungi, like the bacterial TetR-based STF from the Tet-expression system in *A. niger* and *A. fumigatus*[5], the qa-1F-based STF (qa-1F-DBD-VP16, QF) from *Neurospora crassa* in *P. rubens*[7], the bacterial Bm3R1-based STF (Bm3R1-VP16) in *A. niger*, *T. reesei,* and several yeasts[6], as well the Gal4-DBD and LexA-DBD which are frequently used in synthetic expression systems. In Aspergilli the often-utilized Tet-On/Tet-Off system provides precise, reversible, and efficiently controlled gene expression using rtTA and rTA STFs respectively. With the Tet-On system, induced gene activation can be achieved in a titratable manner by addition of the tetracycline derivative doxycycline, whereas induced repression can be achieved using the tetracycline-controlled transactivator (tTA) component to quantitatively reduce gene expression using the Tet-Off system.[5] The Fungal Modular Cloning Toolkit contains a collection of DNA-binding domains (from the qa-1F, Gal4 LexA, TetR transcription factors), and transcriptional activation domains (from the qa-1F, Gal4, B42 transcription factors), VP16 and its four tandem repeats VP64, the tripartite activator VPR (VP64-p65-Rta) as well as histone acetyltransferases (p300core, Rtt109).

CRISPR elements

Next to STFs, catalytically inactive CRISPR-Cas proteins can provide new alternatives for the delivery of transcriptional regulators to the target. The CRISPR/Cas9-based systems require the expression of both the Cas protein and a locus-specific sgRNA in the host organism. The toolkit provides entry vectors for both catalytically active (*spCas9*) and dead (*dSpCas9*) Cas9 versions from the *S. pyogenes*, being the most widely applied Cas protein in filamentous fungi. Catalytically active Cas9 provides opportunities for genome editing, whereas dCas9 can be applied for delivering transcriptional regulators to a desired genomic locus through protein fusion of regulator domains. CRISPRa (activation) and CRISPRi (interference) can provide a genome-editing-free alternative for transcriptional activation and repression, respectively. In comparison with

using STFs, CRISPRa/i tools can provide genome-editing-free transcriptional regulation in filamentous fungi, guiding the regulator to the desired genomic locus, resulting in transcriptional activation (dCas9-VP64 and dCas9-VP64-p65-Rta "VPR")[13,14] or epigenome editing (dCas9-p300)[15]. For CRISPR sgRNA delivery the toolkit provides various options. A sgRNA "plug-and-play" transcription unit carrying (level 1) vector is included, in which the transcript is under control of the *gpdA* RNA polymerase II (Pol II) promoter results in a transcript which is self-cleaved using the hammerhead and hepatitis delta virus ribozymes flanking the sgRNA (HH-sgRNA-HDV).[14] Ribozyme-based sgRNA delivery is widely used in filamentous fungi[18], as it only relies on an established promoter in the host, and ribozymes sequences that work across multiple species. Although the delivery of the ribosomes-self-cleaved sgRNAs is shown to work in numerous fungal applications, in some cases RNA polymerase III (Pol III) transcribed sgRNA delivery could be advantageous, as the created transcript does not need further processing.[18,31]

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Therefore, entry vectors are provided containing a collection of Pol III promoters and corresponding terminators (tRNA-Met, tRNA-Leu, U6, and U3) established in *P. rubens*[28], as well as sgRNA transcription units using tRNA promoters (tRNA-Arg and tRNA-Pro) established in *A. niger*[31] (Table 1). To assemble a functional transcription unit, the latter utilizes the Esp3I restriction enzyme for sgRNA target sequence insertion into the sgRNA transcription unit, the former ones are provided as entry vectors (Figure S1). Two previously established AMA1-based fungal CRISPR vectors with terbinafine and phleomycin markers are also part of this toolkit: pLM-AMA-18.0 for CRISPR-based transcriptional activation and pLM-AMA-15.0 for CRISPR-based genome editing in *P. rubens*, both with a blue/while selection-aided user-friendly sgRNA "plug-and-play" module to aid rapid library construction.[14] The toolkit provides a collection of commonly used transcriptional activation domains (VP16, VP64, VPR), histone acetyltransferases (p300core, Rtt109) and a collection of fluorescent reporters for possible fusion variations.

Fluorescent reporters

Fluorescent reporters are often used to validate genetic circuits, protein expression, and localization through fusions. This toolkit provides a collection of CDSs of fluorescent and bioluminescent reporters (GFP, DsRed, dTomato, mCherry, YFP, BFP, firefly luciferase), with nuclear localization sequence (NLS) or serine-lysine-leucine peroxisomal localization (SKL) or without any localization tags, established in *Aspergillus* and *Penicillium* species (Table 1). Reporters can be used to demonstrate functionality of genetic circuits or as fusion proteins to validate the expression of the gene of interest.

Selection markers

The toolkit contains a collection of the most commonly used fungal selection markers (*ergA, amdS, pyrG, ble, hph, sdh2, hisB*) as entry vectors. For DNA sources of the markers and their established applications see Table 1. Overexpression of the native squalene epoxidase (*ergA*) gene has been shown to provide resistance against terbinafine in a broad range of fungi, as well as in *Penicillium*. In acetamidase activity lacking *Aspergillus*, *Trichoderma,* and *Penicillium* species, overexpression of the acetamidase (*amdS*) gene provides selection on media, containing acetamide as a sole nitrogen source that can be counter selected using fluoroacetamide. The orotidine 5'-phosphate decarboxylase (*pyrG*) gene from *A. oryzae* is widely applied in Aspergilli, with examples in *Penicillium* and *Neurospora*, as a strong, recyclable, auxotrophic selection marker which can be counter selected using 5-fluoroorotic acid or fully supplemented using uracil or uridine. Overexpression of the bacterial resistance genes as phleomycin (*ble*) or the hygromycin B phosphotransferase (*hph*) provides selection in numerous *Aspergillus*, and *Penicillium* strains as well as in *N. crassa* for phleomycin (glycopeptide antibiotic of the bleomycin family) or hygromycin (aminoglycosidic antibiotic) respectively. The succinate dehydrogenase (*sdh2*) gene from *A. niger* is also included, with a single histidine-to-leucine point mutation in the third cysteine-rich cluster (H269L), which showed to play a role in conferring resistance to the fungicide carboxin in *A. flavus*. After generating a histidine-

auxotrophic strain, delivering the key gene of histidine biosynthesis can provide selection. For the creation of such strains, the toolkit provides entry vectors on the native *hisB* genes from *A. niger* and *P. rubens*.

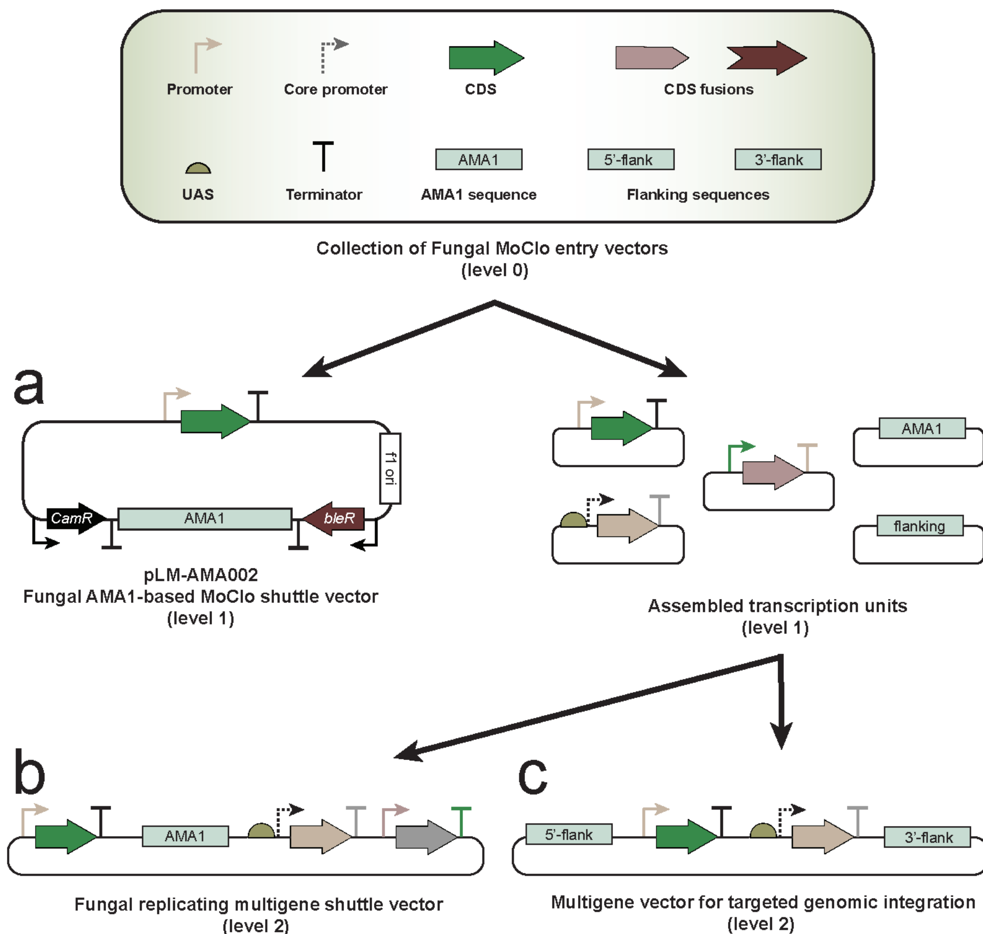

Figure 2. Transcription unit construction using the MoClo system and delivery platforms. Schematic representation of the recombination and assembly of the MoClo entry vectors into transcription units. Transcription units can be assembled into (a) fungal shuttle vectors or (b) into multigene constructs which can be delivered as AMA1-based episomal vectors or (c) via genomic integration by homologous recombination.

Several options exist for the introduction of assembled transcription units in fungi; if the assembled constructs include the AMA1 sequence it can be delivered as an episomal vector (Figure 2a-b) or multigene constructs can be integrated to a genomic locus using homologous flanking sequences (Figure 2c). In the toolkit, fungal shuttle vectors with an AMA1 sequence are included. The AMA1 sequence supports autonomous plasmid replication in numerous filamentous fungi, as well as flanking regions for homologous recombination-based genomic integration into *P. rubens* at the frequently used penicillin (Pc21g21370-Pc21g21390) and PKS17 (Pc21g16000) loci. A 50% shorter version of the AMA1 sequence is also provided on a MoClo entry vector, which can be incorporated in complex MoClo language-based constructs. This truncated sequence can be amplified by PCR and showed transient vector propagation while maintaining selection pressure, and without selection a more rapid loss of the vector was detected, compared to a full-size AMA1 vector in *A. niger.*[25] As this sequence is integrated on a MoClo entry vector, it is possible to incorporate it into a MoClo multigene construct (level 2), turning the originally bacterial vector into a fungal replicating episomal vector (Figure 2b). Fungal shuttle vectors can be assembled in *E. coli* and delivered into *Aspergillus*, *Penicillium*, potentially into other fungi in the *Aspergillaceae* family, or any other AMA1- and selection-marker-compatible fungal host. The vector allows rapid assembly and validation of transcription units, providing alternatives for genomic integration (Figure 2c).

For this toolkit, a shuttle vector (pLM-AMA002) analogous to a MoClo system "level 1" backbone was built, thus providing a MoClo entry vector compatible, fungal transcription unit delivery platform (Figure 2a, Figure 3). As the assembly follows the MoClo language[19], the vector uses BsaI restriction enzyme generated GGAG and CGCT fusion sites for receiving the compatible MoClo entry vectors. The fungal shuttle vector additionally contains a *lacZα* fragment, which is replaced during the assembly of the transcription unit, allowing for a convenient blue/white screening of successful clones. Created transcription unit carrying vectors can directly be transformed into fungal hosts, using phleomycin as a selection marker. To test our MoClo-adapted and AMA1-

based fungal shuttle vector for expressing a gene of interest, a transcription unit was assembled expressing a fusion protein of the catalytically dead Cas9 protein (dSpCas9) from *S. pyogenes* and an eGFP-NLS (green fluorescent protein with SV40 nuclear localization) reporter. The genetic parts were rapidly assembled into a transcription unit on the pLM-AMA002 fungal shuttle vector, through the first two steps (level 0 construction and level 1 assembly) of the MoClo assembly (Figure 3a). The restriction-ligation-based assembly resulted in an AMA1 vector expressing a direct fusion of dSpCas9-eGFP-NLS, driven by a constitutive promoter. The created vector was delivered to *P. rubens* and the expression of the protein fusion was validated using fluorescence microscopy, showing expression of nucleus localized GFP expression (Figure 3b). The construction of this expression platform required the integration of the coding sequence (CDS) of the gene of interest into the appropriate position-predetermined MoClo entry vector. As numerous entry vectors can be utilized from the toolkit, the assembly and validation time of a transcription unit can be significantly reduced. After successful validation of additional new entry vectors no more sequencing is required later assembly steps. With the high efficiency of the MoClo assembly, transcription units can be rapidly assembled in one single cloning step. Meanwhile, multigene genetic circuits can be constructed in two cloning steps (carrying up to 7 transcription units per assembly).[19]

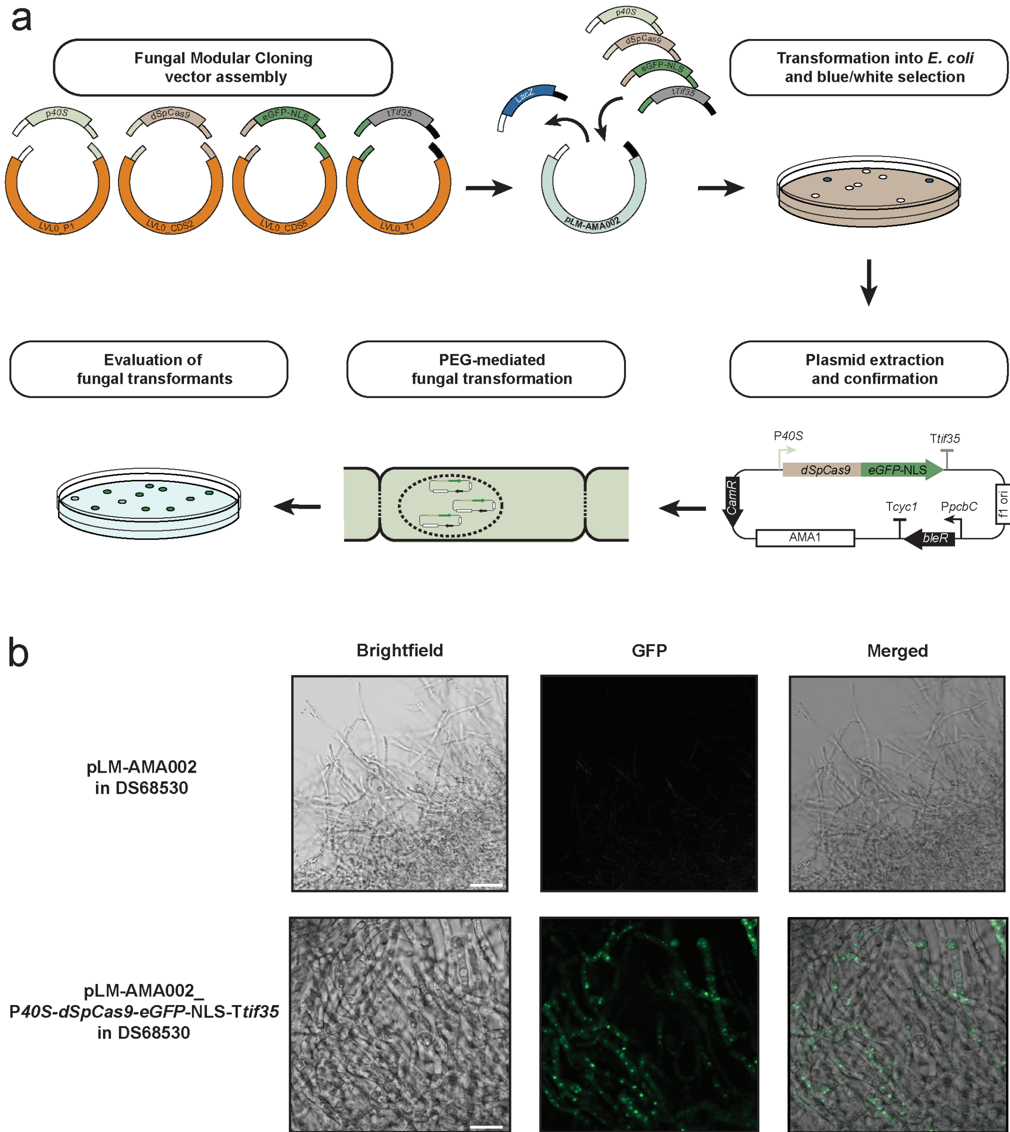

Figure 3. Transcription unit assembly from MoClo entry vectors on pLM-AMA002 fungal shuttle vector and delivery to filamentous fungi. (a) Schematic representation of the assembly of MoClo entry vectors into a single transcription unit delivered to *P. rubens* on the pLM-AMA002 fungal shuttle vector. (b) Fluorescence microscopy imaging of filaments of *P. rubens* strain carrying pLM-AMA002 with the *dSpCas9-eGFP-NLS* transcription unit, showing protein expression of the fluorescently labelled gene product. Scale bars represent 20 μm.

Taken together, this Fungal Modular Cloning Toolkit aims at accelerating synthetic biology for filamentous fungi by providing essential, ready-to-use genetic parts for rapid construction of genetic circuits as well as CRISPR components for more efficient genome engineering and providing aid in biotechnological exploitation. This toolkit provides genetic parts for flexible and efficient assembly of genetic circuits for filamentous fungi in the form of 96 MoClo entry vectors and assembled transcription units. It is a collection of promoters (constitutive, inducible), terminators, activator- and DNA-binding-domains of transcription factors, fluorescent reporters, fungal selection markers, as well as CRISPR proteins (SpCas9, dSpCas9) which are applicable for CRISPR-based applications. All vectors are build using the MoClo synthetic biology language, which allows the user to assemble numerous transcription units on one single plasmid, that can be later delivered to the desired host organism by various delivery methods. To further accelerate the testing of functional transcription units, genetic parts are included, which were tested in the community and shown to be interchangeable between different fungal strains. This collection of fungal genetic parts was created using the "MoClo Toolkit"[19], therefore this toolkit (or an equivalent version of it) is needed for the incorporation of new genetic parts for further novel assemblies, unless these parts are delivered into the assembly as vector-free DNA fragments. As most of the genetic parts of the toolkit were tested in *A*. *nidulans*, *A*. *niger,* and *P*. *rubens* strains (Table 1), this toolkit aims for compatibility with strains in the *Aspergillaceae* family, but assuming functionality in other filamentous fungal strains. Positions of the modular entry vectors in a transcription unit assembly is represented together with location identifiers on Figure 1. Complete vector sequences are available as Genebank files in Supp. File S1 and available on Addgene as the "Fungal Toolkit for Modular Cloning (FTK)" under Kit ID 1000000191.

Methods

Chemicals, reagents and oligodeoxyribonucleotides and cloning

All medium components and chemicals were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands) or Merck (Darmstadt, Germany). Oligodeoxyribonucleotide primers were obtained from Merck. Enzymes were obtained from Thermo Fisher Scientific (Waltham, MA) unless otherwise stated. For the design of nucleic acid constructs, *in-silico* restriction cloning and inspection of Sanger sequencing results, SnapGene (GSL Biotech) was used. PCR amplifications were conducted using KAPA HiFi HotStart ReadyMix (Roche Diagnostic, CH). Templates for PCR amplifications were acquired from various sources (Table 1) or were ordered as synthetic DNA fragments from Thermo Fisher Scientific (Waltham, MA). All internal BpiI and BsaI cloning sites (and in some cases DraIII and Esp3I) were removed during cloning from the DNA fragments, and these sequences were manually curated for frequent codons in *P. rubens*. All vectors were constructed using the MoClo assembly system and protocol.[19] The receiver backbones (established in the Modular Cloning assembly[19]) used for constructing the genetic parts containing entry vectors are highlighted in Figure 1b. As the linker sequences between the genetic parts in the transcription unit are based on the standard MoClo language (Figure 1a), the parts are compatible with modular systems which are using this linker system. Correctly assembled plasmids were identified with blue-white screening and confirmed by sequencing. The transcription unit expressing SpCas9-eGFP-NLS on a fungal shuttle vector (pLM-AMA002_P*40s*-dSpCas9-eGFP-NLS-T*tif35*) was assembled using a mixture of 30 fmol of each entry vectors (P*40s* An0465 (P1), dSpCas9(m2) (CDS2), eGFP-NLS (CDS5), T*tif35* (T1) and the backbone vector pLM-AMA002.

The 50% shorter AMA1 sequence[25] was created by PCR, and was integrated into a MoClo entry vector. The autonomously replicating shuttle vector, carrying the AMA1 sequence, was based on pDSM-JAK-109 backbone where the Pg*pdA*-*DsRed*-SKL-T*penDE* transcription unit was removed using the BspTI and NotI restriction enzymes. The linear vector was treated with the Klenow Fragment of

DNA polymerase I and self-ligated into a circular vector using the T4 DNA Ligase according to the instructions of the manufacturer, creating a new AMA1 vector without DsRed expression. This vector was cloned with a removable *LacZ* gene cloning site using BspTI, based on the "level 1" receiver backbones of the MoClo system, creating pLM-AMA002.

Fungal strains, transformation and cultivation

Cultivation of fungal and bacterial strains, media composition, protoplast generation and fungal transformation using phleomycin marker was carried out as described previously.[14] A list of fungal strains created in this study with corresponding transformed donor DNA can be found in Supp. Table S1.

Fluorescence microscopy

Transformants were further cultivated after transformation on phleomycin (50 μg/ml) supplemented transformation solid medium for 5 days and were examined using fluorescence microscopy. A small amount of hyphae was taken from the peripheral zone of the colonies and suspended in phosphate-buffered saline (58 mM Na_2HPO_4; 17 mM NaH_2PO_4; 68 mM NaCl, pH 7.3). Confocal imaging was performed on a Carl Zeiss LSM800 confocal microscope using 20x objective and ZEN 2009 software (Carl Zeiss, Oberkochen, Germany). The GFP signal was visualized by excitation with a 488 nm argon laser (Lasos Lasertechnik, Jena, Germany) and emission was detected using a 509 nm band-pass emission filter.

Vector name	Add-gene ID	Part type	Unit description	Recipient MoClo backbone	Template	Source
pFTK001	171273	P1	Pact Pc20g11630 promoter	pICH41295	*P. rubens* DS54468	27
pFTK002	171274	P1	PgndA An11g02040 promoter	pICH41295	*A. niger* N402	7,27
pFTK003	171275	P1	Patp9 An04g08190 promoter	pICH41295	*A. niger* N402	27
pFTK004	171276	P1	PgpdA ANIA_08041 promoter	pICH41295	pDONR221-AMDS	27
pFTK005	171277	P1	Ptef1 ANIA_04218 promoter	pICH41295	pFC334 (Addgene #87846)	32
pFTK006	171278	P1	Ptef EF1-subunit ANIA_02063 promoter	pICH41295	*A. nidulans* FGSC A4	33
pFTK007	171279	P1	PfraA An16g04690 promoter	pICH41295	*A. niger* N402	5

Vector name	Add-gene ID	Part type	Unit description	Recipient MoClo backbone	Template	Source
pFTK008	171280	P1	Poat1 Pc18g03600 promoter	pICH41295	*P. rubens* Wisconsin 54–1255	34
pFTK009	171281	P1	PglaA An03g06550 promoter	pICH41295	pEBA520	5
pFTK010	171282	P1	PxlnA ANIA_03613 promoter	pICH41295	*A. nidulans* FGSC A4	35
pFTK011	171283	P1	PpcbAB Pc21g21390 promoter	pICH41295	*P. rubens* DS54468	27
pFTK012	171284	P1	P40s An0465 promoter	pICH41295	pDSM-JAK-108	7,36
pFTK013	171285	P1	PpcbC Pc21g21380 promoter	pICH41295	*P. rubens* DS54468	27
pFTK014	171286	P1	PAnHisB AN6536.2 promoter	pICH41295	*A. niger* N402	37
pFTK015	171287	P1	PpyrG AO090011000868 promoter	pICH41295	pMF21.1	38
pFTK016	171288	P1	1x TetO UAS + CPgpdA (fused)	pICH41295	pVG2.2	5
pFTK017	171289	P1	4x TetO UAS+ CPgpdA (fused)	pICH41295	pVG2.2	5
pFTK018	171290	P1	6x TetO UAS + CPgpdA (fused)	pICH41295	pVG2.2	5
pFTK019	171291	P1	10x TetO UAS + CPgpdA (fused)	pICH41295	pVG2.2	5
pFTK020	171292	P1	PalcA synt_NoCrea (ANIA_08979) promoter	pICH41295	*A. nidulans* FGSC A4	39
pFTK021	171293	P1	Pu3 hom., Putp25, *P. rubens* Pol-III promoter	pICH41295	*P. rubens* DS54468	28
pFTK022	171294	P1	Pu6 hom., *P. rubens* Pol-III promoter	pICH41295	*P. rubens* DS54468	28
pFTK023	171295	P1	PtRNA[Met] *P. rubens* Pol-III promoter	pICH41295	*P. rubens* DS54468	28
pFTK024	171296	P1	PtRNA[Leu] *P. rubens* Pol-III promoter	pICH41295	*P. rubens* DS54468	28
pFTK025	171297	P1	CPpcbC Pc21g21380 (no UAS) core promoter	pICH41295	*P. rubens* Wisconsin 54–1255	7
pFTK026	171298	P1	CPnirA AN0098 (no UAS) core promoter	pICH41295	*A. nidulans* FGSC A4	7
pFTK027	171299	P1	CPura3 YEL021W (no UAS) core promoter	pICH41295	*S. cerevisiae* CEN.PK113-7D	7
pFTK028	171300	P2	1xQUAS UAS (for fusion)	pAGM1251	synthetic DNA	7,40
pFTK029	171301	P2	5xQUAS UAS (for fusion)	pAGM1251	synthetic DNA	7,40
pFTK030	171302	P2	11xQUAS UAS (for fusion)	pAGM1251	synthetic DNA	7,40
pFTK031	171303	P2	5xLexA_UAS UAS (for fusion)	pAGM1251	synthetic DNA	41
pFTK032	171304	P3	CPpcbC Pc21g21380 core promoter (for fusion)	pAGM1276	*A. nidulans* FGSC A4	7
pFTK033	171305	P3	CPnirA AN0098 core promoter (for fusion)	pAGM1276	*S. cerevisiae* CEN.PK113-7D	7
pFTK034	171306	P3	CPura3 YEL021W core promoter (for fusion)	pAGM1276	*A. niger* N402	7
pFTK035	171307	CDS1	ergA Pc22g15550 terbinafine, selection marker	pICH41308	*P. rubens* DS54468	42
pFTK036	171308	CDS1	amdS ANIA_08777 acetamidase, selection marker	pICH41308	pDONR221-AMDS	29
pFTK037	171309	CDS1	bleoR phleomycin, selection marker	pICH41308	pDSM-JAK-109	29,36

Vector name	Add-gene ID	Part type	Unit description	Recipient MoClo backbone	Template	Source
pFTK038	171310	CDS1	hph hygromycin selection marker (hygR)	pICH41308	pAN7.1	43
pFTK039	171311	CDS1	pyrG AO090011000868 Orotidine 5′-phosphate decarboxylase, selection marker	pICH41308	pMF21.1	38
pFTK040	171312	CDS1	sdh-H85L An14g04400 succinate dehydrogenase, selection marker	pICH41308	*A. niger* N402	44
pFTK041	171313	CDS1	hisB Pc20g11690 histidine, selection marker	pICH41308	*P. rubens* DS54468	37
pFTK042	171314	CDS1	hisB AN6536.2 histidine, selection marker	pICH41308	pSE1.6	37
pFTK043	171315	CDS1	eGFP fluorescent reporter	pICH41308	pLM2_30 (Addgene #154222)	7
pFTK044	171316	CDS1	eGFP-NLS fluorescent reporter	pICH41308	pLM2_30 (Addgene #154222)	7
pFTK045	171317	CDS1	eGFP-SKL fluorescent reporter	pICH41308	pLM2_30 (Addgene #154222)	7
pFTK046	171318	CDS1	DsRed.T1 fluorescent reporter	pICH41308	pDSM-JAK-109	7,27,36
pFTK047	171319	CDS1	DsRed-NLS fluorescent reporter	pICH41308	pDSM-JAK-109	7,27,36
pFTK048	171320	CDS1	DsRed.T1-SKL fluorescent reporter	pICH41308	pDSM-JAK-109	27,36,45
pFTK049	171321	CDS1	mCherry fluorescent reporter	pICH41308	pURA3_1147651cp_ mCherry	6
pFTK050	171322	CDS1	dTomato fluorescent reporter	pICH41308	pMF30.1	46
pFTK051	171323	CDS1	eBFP fluorescent reporter	pICH41308	pLM2_30 (Addgene #154222) with Y66H/ Y145F mutations	7
pFTK052	171324	CDS1	Firefly luciferase reporter	pICH41308	pVG4.1	5
pFTK053	171325	CDS1	alcR ANIA_08978 transcriptional activator	pICH41308	*A. nidulans* FGSC A4	39
pFTK054	171326	CDS1	QS (QA-1S) codon optimized, quinic acid repressor	pICH41308	pAC-Qsco, (Addgene #46106)	40
pFTK055	171327	CDS1	rtTA2S-M2 (TetR-3xVP16) transcriptional activator	pICH41308	pVG2.2	5
pFTK056	171328	CDS1	SpCas9-NLS	pICH41308	pYTK036 (Addgene #65143)	14
pFTK057	171329	CDS1	dSpCas9(m4)-VPR-NLS	pICH41308	pYTK036 (Addgene #65143), pAG414GPD (Addgene #63801)	14,47
pFTK058	171330	CDS2	QF DBD from QA-1F (for fusion)	pICH41258	pAC-7-QFBDAD (Addgene #46096)	40
pFTK059	171331	CDS2	LexA DBD (for fusion)	pICH41258	FRP718_PACT1(-1-520)-LexA-ER-haB42-TCYC1 (Addgene #58431)	48
pFTK060	171332	CDS2	Gal4D BD (for fusion)	pICH41258	*S. cerevisiae* CEN.PK113-7D	49
pFTK061	171333	CDS2	SpCas9 (for fusion)	pICH41258	pYTK036 (Addgene #65143)	14,21

Vector name	Add-gene ID	Part type	Unit description	Recipient MoClo backbone	Template	Source
pFTK062	171334	CDS2	dSpCas9(m2) (for fusion)	pICH41258	pYTK036 (Addgene #65143)	14,21
pFTK063	171335	CDS2	dSpCas9(m4) (for fusion)	pICH41258	pYTK036 (Addgene #65143)	14,21
pFTK064	171336	CDS3	QF AD from QA-1F (for fusion)	pAGM1299	pAC-7-QFBDAD (Addgene #46096)	7,40
pFTK065	171337	CDS3	B42 AD (for fusion)	pAGM1299	FRP718_PACT1(-1-520)-LexA-ER-haB42-TCYC1 (Addgene #58431)	48
pFTK066	171338	CDS3	Gal4 AD (for fusion)	pAGM1299	*S. cerevisiae* CEN.PK113-7D	49
pFTK067	171339	CDS3	VP16 AD (for fusion)	pAGM1299	pVG2.2	5,49
pFTK068	171340	CDS3	VP64 AD (for fusion)	pAGM1299	pcDNA-dCas9-VP64 (Addgene #47107)	47
pFTK069	171341	CDS4	eGFP-NLS fluorescent reporter (for fusion)	pAGM1301	pLM2_30 (Addgene #154222)	7
pFTK070	171342	CDS4	eYFP-NLS fluorescent reporter (for fusion)	pAGM1301	pLM2_30 (Addgene #154222) with S65G/V68L/S72A/T203Y mutations	7
pFTK071	171343	CDS5	eGPF-NLS fluorescent reporter (for fusion)	pICH41264	PX458 (Addgene #48138)	7
pFTK072	171344	CDS5	p300core HAT AD, Homo sapiens E1A binding protein p300 (for fusion)	pICH41264	pcDNA-dCas9-p300 (Addgene #61357)	15,47,50
pFTK073	171345	CDS5	RTT109 HAT AD (for fusion)	pICH41264	*S. cerevisiae* CEN.PK113-7	51
pFTK074	171346	CDS5	VPR -NLS AD (for fusion)	pICH41264	pAG414GPD (Addgene #63801)	47
pFTK075	171347	T1	TamdS ANIA_08777 terminator	pICH41276	pDONR221-AMDS	29
pFTK076	171348	T1	Ttif35 Pc22g19890 terminator	pICH41276	pDSM-JAK-108	7,36
pFTK077	171349	T1	TpenDE Pc21g21370 terminator	pICH41276	*P. rubens* Wisconsin 54–1255	36
pFTK078	171350	T1	TxlnA ANIA_03613 terminator	pICH41276	*A. nidulans* FGSC A4	35
pFTK079	171351	T1	Toat1 Pc18g03600 terminator	pICH41276	*P. rubens* Wisconsin 54–1255	34
pFTK080	171352	T1	Tcyc1 YJR048W terminator	pICH41276	pDSM-JAK-109	36
pFTK081	171353	T1	TactA (Tact1) ANIA_06542 *P. rubens* terminator	pICH41276	pDSM-JAK-108	7,36
pFTK082	171354	T1	Tu3 hom., Tutp25, *P. rubens* Pol-III terminator	pICH41276	*P. rubens* DS54468	28
pFTK083	171355	T1	Tu6 hom., *P. rubens* Pol-III terminator	pICH41276	*P. rubens* DS54468	28
pFTK084	171356	T1	TtRNA[Met] *P. rubens* Pol-III terminator	pICH41276	*A. niger* N402	28
pFTK085	171357	T1	TtRNA[Leu] *P. rubens* Pol-III terminator	pICH41276	*A. niger* N402	28

Vector name	Add-gene ID	Part type	Unit description	Recipient MoClo backbone	Template	Source
pFTK086	171358	TU	P-ANtRNA[Arg21]-sgRNA-dummy-Esp3I, Pol-III sgRNA transcription unit	pICH41331	*A. niger* N402	31
pFTK087	171359	TU	P-ANtRNA[Pro1]-sgRNA-dummy-Esp3I, Pol-III sgRNA transcription unit	pICH41331	*A. niger* N402	31
pFTK088	171360	TU	AMA1 sequence (short), entry vector providing fungal replication	pICH41331	pDSM-JAK-109	25,36
pFTK089	171361	TU	penicillin gene cluster *P. rubens* 5'flanking region	pICH41331	*P. rubens* DS54468	7
pFTK090	171362	TU	penicillin gene cluster *P. rubens* 3'flanking region	pICH41331	*P. rubens* DS54468	7
pFTK091	171363	TU (lvl1)	pks17 Pc21g16000 (conidial pigment biosynthesis) *P. rubens* 5'flanking region	pICH47732 (lvl1)	*P. rubens* DS54468	28
pFTK092	171364	TU (lvl1)	pks17 Pc21g16000 (conidial pigment biosynthesis) *P. rubens* 3'flanking region	pICH47772 (lvl1)	*P. rubens* DS54468	28
pFTK093	171365	TU (lvl1)	sgRNA transcription unit (MoClo lvl1 unit), P-gpdA-HH-sgRNA-HDV-Ttrpc	pICH47761 (lvl1)	pFC334 (Addgene #87846), pLM-AMA18.0 dCas9-VPR (Addgene #138945)	14
pFTK094-LM-AMA002.0	171366	AMA1	pLM-AMA002, fungal shuttle vector with bleoR marker for MoClo entry vectors	n/a	pDSM-JAK-109	36
pFTK095-LM-AMA015.0	171367 / 138944	AMA1	pLM-AMA15.0, CRISPR/Cas9 genome editing with HH-sgRNA-HDV transcription unit, ergA and bleoR fungal markers	n/a	pDSM-JAK-109, pYTK036 (Addgene #65143), pLM-AMA15.0 Cas9 (Addgene #138944)	14
pFTK096-LM-AMA018.0	171368 / 138945	AMA1	pLM-AMA18.0, CRISPRa/ dSpCas9-VPR transcriptional activator with HH-sgRNA-HDV transcription unit, ergA and bleoR fungal markers	n/a	pDSM-JAK-109, pYTK036 (Addgene #65143), pAG414GPD (Addgene #63801, pLM-AMA18.0 dCas9-VPR (Addgene #138945),	14

Table 1. Genetic modules and other vectors in the Fungal Toolkit for Modular Cloning (FTK). Units in the toolkit are described using a Vector name, Addgene ID, a Part type specifying the function of the part (P: promoter; CDS: coding sequence; T: terminator; TU: transcription unit; AMA1: AMA1-sequence based fungal replicating vector), a short description of the vector, its recipient Modular Cloning destination vector, the source of the genetic element and its applications(s).

Abbreviations

MoClo, Modular Cloning; BGC, biosynthetic gene cluster; NRPS, non-ribosomal peptide synthetases; PKS, polyketide synthase; STF, synthetic transcription factor; DBD, DNA-binding domain; UAS, upstream activating sequence; CRISPR, clustered regularly interspaced short palindromic repeats; CAS, CRISPR-associated protein; sgRNA, single guide RNA; AD, activator domain

Author contributions

†:L.M. and C.P. contributed equally to this work. L.M. and C.P. designed and carried out all experiments and wrote the manuscript with critical feedback and help from V.M., R.A.L.B., Y.N. and A.J.M.D.

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Supplementary information

Supplementary File S1. Sequence information of the Fungal Toolkit for Modular Cloning (FTK)

Supplementary File S1 is available online at:
https://www.addgene.org/kits/driessen-modular-cloning-fungi or
https://pubs.acs.org/doi/full/10.1021/acssynbio.1c00260

Fungal strain	Genotype	Transformed donor DNA
P. rubens DS68530	Δ*hdfA* (Δ*ku70*), Δpen-BGC	n/a
DS68530_pLM-AMA002	Δ*hdfA* (Δ*ku70*), Δpen-BGC	pLM-AMA002
DS68530_pLM-AMA002_P40s-dSpCas9-eGPF-NLS-T*tif35*	Δ*hdfA* (Δ*ku70*), Δpen-BGC	pLM-AMA002_P*40s*-dSpCas9-eGPF-NLS-T*tif35*

Table S1. List of fungal strains used in this study and created strains with their corresponding transformed donor DNA

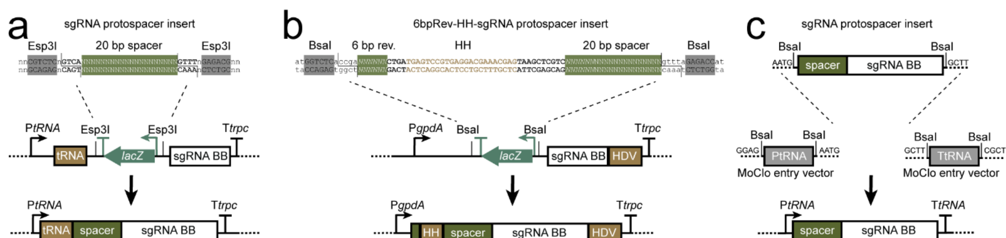

Figure S1. Representation of different sgRNA transcription unit assembly methods. (a) Ligation of the sgRNA target sequence carrying double-stranded-DNA into sgRNA recipient cloning site, using Esp3I restriction sites for PtRNA driven and tRNA-linked transcripts, (b) Ligation of HH-sgRNA (and 6 bp inverted repeat of the spacer sequence) using BsaI sites for P*gpdA* driven HH-sgRNA-HDV-based transcription (c) Ligation of sgRNA sequence using entry vectors and BsaI restriction-ligation-based assembly for creating functional sgRNA transcription units.

CHAPTER 6

Summary

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Summary

Secondary metabolites (SMs) are a diverse group of molecules, naturally produced by plants, bacteria and fungi. SM biosynthesis employs metabolic intermediates from primary metabolism, and the core skeleton of these molecules is commonly produced by nonribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), or combined enzymes. The increasing number of sequenced fungal genomes revealed a vast of natural product biosynthetic gene clusters (BGCs) with unknown produced compounds. However, most of the fungal SM BGCs are transcriptionally silent under laboratory growth conditions, and the signal or stimulus triggering the transcriptional activation of these clusters is often unknown, rendering these BGC "cryptic". *Penicillium rubens* (previously identified as *P. chrysogenum*) contains 33 core genes (10 NRPS, 20 PKS, 2 hybrid NRPS–PKS, and 1 dimethyl-allyl-tryptophan synthase) responsible for SM production and –despite being the most studied member of its genus with 354 species– the product of more than half of these BGCs is unknown. Obviously, considering the enormous diversity of the fungal kingdom and the notion that BGCs are widespread, characterized SMs so far represent only the tip of the ice-berg. With the advent of synthetic biology novel applications and tools have been developed for fungi, aiming at the transcriptional activation of cryptic BGCs. **Chapter 1** provided an overview of regulation of SM BGCs, and a comparison between conventional and novel approaches for transcriptional reprogramming. These include synthetic transcription factors (STFs), assembly of artificial transcription units, gene cluster refactoring, fungal shuttle vectors and platform strains for heterologous expression.

Chapter 2 described the construction of orthogonal, synthetic control devices for transcriptional regulation in *P. rubens*, using a STF and various synthetic promoters. We constructed a STF from the fusion of the QF DNA-binding domain of the TF of the quinic acid gene cluster of *Neurospora crassa* (qa-1F-DBD) and the activation domain of VP16 from herpes simplex virus (VP16-AD), tagged with a green fluorescent protein (GFP) together with an SV40 nuclear localization signal

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(NLS). As the qa-1F-DBD targets and binds to its unique quinic acid upstream activating sequences (QUASs) in a promoter, integrating these sequences in promoters results in elevated gene expression. With the fusion of QUASs and core promoters (CPs) from various sources (*P. rubens*, *Aspergillus nidulans*, and *Saccharomyces cerevisiae*) synthetic promoters were constructed. BGCs may demand an expression individually tuned for each gene, and this is supported with the elements chosen from this system. The strength of the transcriptional control showed scalability by changing different modular elements of the expression. The transcriptional control devices were characterized with respect to three of their main components: expression of the STFs, upstream activating sequences, and the affinity of the DNA binding domain of the TF to the upstream activating domain. The regulatory systems were evaluated in micro bioreactor cultivations using a red fluorescent reporter (DsRed-T1-SKL) representing the gene of interest. By integrating the STF and QUAS elements into the native CPs of the penicillin BGC in *P. rubens*, the production was taken under the control of the STF and the penicillin titer was dependent on the number of integrated QUAS elements.

Chapter 3 described the construction of a non-integrative, fungal vector-based, *in vivo* expressed CRISPR/Cas9 delivery system for filamentous fungi. Fungal promoters were evaluated for the *in vivo* expression of CRISPR components in a non-homologous end-joining (NHEJ) DNA-repair deficient *P. rubens* strain. Cas9 protein expression in *P. rubens* was visualized using a GFP-NLS tag, and the Cas9-eGFP-NLS transcription unit was delivered on a Modular Cloning-compatible fungal shuttle vector (described in **Chapter 5**). The constructed CRISPR/Cas9 vectors were evaluated for genome editing using a fluorescent reporter knock-out experiment, using a constitutively DsRed-expressing strain (constructed in **Chaper 2**). A native polymerase III tRNA[Serine] promoter and the gpdA polymerase II promoter in combination with (HH and HDV) self-cleaving ribozyme sequences were successfully identified for *in vivo* sgRNA delivery in *P. rubens*. We choose the HH-sgRNA-HDV expression system for further work, as it has

been shown to be functional in various fungal species. The established CRISPR/Cas9 fungal shuttle vector (pLM-AMA-15) contains phleomycin and terbinafine resistance markers, a Cas9 expression cassette, and a sgRNA acceptor site which facilitates rapid assembly of functional HH-sgRNA-HDV expression units and library construction. The CRISPR vector and donor DNA with short homologous flanking regions (106 bp) was used for marker-free knock-out of an N-terminal pseudo condensation domain of the *pcbAB* gene (Pc21g21390, ACV synthetase), which revealed to be critical for the functionality of the enzyme. As the vector backbone supports autonomous replication in several filamentous fungal species, and as we use established genetic elements to deliver CRISPR components, we expect this CRISPR/Cas9 system is transferable to other fungal species.

Chapter 4 describes the development and application of a CRISPR-based transcriptional activation tool (CRISPRa/dCas9-VPR) for transcriptionally silent genes in *P. rubens*. The dCas9-VPR and the self-cleaved sgRNAs were delivered to *P. rubens* on a fungal replicating vector, which is compatible with several filamentous fungal species, and its modular sgRNA insertion-site allows rapid library construction (established in **Chapter 3** for genome editing). The CRISPRa/Cas9 vector (pLM-AMA-18) was evaluated for transcriptional activation of the penDE core promoter-driven DsRed gene, integrated into the penicillin-locus of *P. rubens*. This core promoter has been established in **Chapter 2** to be insufficient to express the fluorescent reporter on its own, instead depending on the presence of a QUAS-binding STF in the promoter. The constructed CRISPRa/dCas9-VPR vectors were designed to deploy sgRNAs which are binding in the close proximity of the transcription starts site of the gene of interest. When active CRISPRa/dCas9-VPR vectors were transformed into *P. rubens*, fluorescence microscopy showed a clear increase in fluorescence with 3 out of 6 tested spacers, compared to a non-sgRNA expressing control. To show that the CRISPRa vector can upregulate an entire silent BGC and induce metabolite production, we targeted the macR TF of the endogenous, transcriptionally silent macrophorin biosynthesis cluster in *P. rubens*. Out of the 20 spacers tested covering the complete promoter of *macR*,

two resulted in transcriptional activation of the macrophorin BGC and related secondary metabolite production. The established CRISPRa system provided a rapid and convenient way for activation of transcriptionally silent genes without the need of genetic engineering of the host.

Chapter 5 describes the construction of a genetic toolkit for synthetic biology applications for filamentous fungi. This Fungal Toolkit (FTK) for Modular Cloning contains 96 ready-to-use, characterised genetic elements which can be assembled in various combination using the Golden Gate-based Modular Cloning assembly. The collection contains natural and synthetic promoters (constitutive and inducible), terminators, fluorescent reporters, and selection markers. Furthermore, there are regulatory and DNA-binding domains of transcriptional regulators and components for the construction of STFs or implementing different CRISPRa/i-based technologies. Genetic parts can be assembled into complex multipartite assemblies and delivered through genomic integration or expressed from an AMA1-sequence-based, fungal-replicating shuttle vector. The entry vectors encoding P*40s*, dSpCas9, eGFP-NLS and T*tif35* were assembled on a Modular Cloning-compatible fungal shuttle vector (pLM-AMA002). The constructed transcription unit was delivered to *P. rubens*. The expression of the nuclease-dead Cas9 protein with a green fluorescent protein tag was validated using fluorescence microscopy. Furthermore, the Fungal Modular Cloning Toolkit was applied to construct 22 synthetic transcriptional control device (**Chapter 2**), transcription units for CRISPR/Cas9 genome editing (**Chapter 3**) and for CRISPRa applications (**Chapter 4**). With this toolkit, synthetic transcription units with characterised promoters, fusion proteins, or synthetic transcriptional regulation devices can be more rapidly assembled in a standardized and modular manner for novel fungal cell factories.

Taken together, we have developed a fungal expression system, where genes of a BGC can be expressed orthogonally under the control of a STF. We constructed a fungal shuttle vector supporting rapid library construction for CRISPR/Cas9-

mediated genome editing. We established a novel CRISPR-based transcriptional activation platform (CRISPRa/Cas9-VPR), which provided transcriptional activation of a transcriptionally silent BGCs in *P. rubens*. As synthetic biology allows the development of new ways for transcriptional regulation, establishing these tools and methods for filamentous fungi –organisms which are naturally wired for secondary metabolite production– is key for fully exploiting their biosynthetic potential. All the tools developed in this thesis aim to unlock the hidden potential of fungal cryptic BGC and accelerate synthetic biology for filamentous fungi serving the academic and industrial fungi community worldwide.

Samenvatting

Secondaire metabolieten (SMs) zijn een diverse groep van moleculen die in de natuur geproduceerd worden door planten, bacteriën en schimmels. SM-biosynthese gebruikt metabole tussenproducten van het primair metabolisme en het kernkelet van deze moleculen wordt gewoonlijk geproduceerd door non-ribosomale peptide synthases (NRPSs), polyketide synthases (PKSs) of gecombineerde enzymen. Het toenemende aantal schimmelgenomen waarvan de sequentie is bepaald, onthulde ene groot aantal biosynthetische genclusters (BGC's) van natuurlijke producten met onbekende structuren en activiteiten. De meeste schimmel SM BGC's zijn echter zwijgend onder laboratiriums groeiomstandigheden en het signaal of de stimulus dat de transcriptie factor, van deze clusters, activeert is vaak onbekend, waardoor deze BGCs vaak als ''cryptisch'' worden beschouwd. *Penicillium rubens* (voorheen bekend als *P. chrysogenum*) bezit 33 kern genen (10NRPS, 20 PKS, 2 hybride NRPS–PKS, en 1 dimethyl-allyl-tryptofaansynthase) die verantwoordelijk zijn voor SM- productie. Ondanks dat dit de meest onderzochte soort is, in een genus van 354 soorten, is meer van de helft van de BGCs onbekend. Gezien de enorme diversiteit van het schimmelrijk en het gegeven dat BGCs wijd verspreid zijn over soorten, geeft duidelijk weer dat de gekarakteriseerde SMs tot nu toe enkel het topje van de ijsberg zijn. Met de komst van synthetische biologie zijn nieuwe toepassingen en hulpmiddelen ontwikkeld voor schimmels, gericht op de transcriptionele activering van cryptische BGC's.

Hoofdstuk 1 geeft een overzicht van de regulatie van SM BGC's, en een vergelijking tussen conventionele en nieuwe benaderingen voor transcriptionele herprogrammering. Deze omvatten synthetische transcriptiefactoren (STF's), een verzameling van kunstmatige transcriptie-eenheden, gencluster-refactoring, schimmelshuttlevectoren en platformstammen voor heterologe expressie.

Hoofdstuk 2 beschrijft de constructie van orthogonale, synthetische controle systemen voor transcriptionele regulatie in *P. rubens*, met behulp van een STF en

verschillende synthetische promoters. We hebben een STF geconstrueerd uit de fusie van het QF-DNA-bindende domein van de TF van het kininezuur gencluster van *Neurospora crassa* (qa-1F-DBD) en het activeringsdomein van VP16 uit herpes simplex-virus (VP16-AD), getagd met een groen fluorescerend eiwit (GFP) samen met een SV40 nucleair lokalisatiesignaal (NLS). Aangezien de qa-1F-DBD bindt aan zijn unieke stroomopwaartse activerende kininezuursequenties (QUAS's) in een promotor, resulteert het integreren van deze sequenties in promotors in verhoogde genexpressie. Met de fusie van QUAS's en kernpromotors (CP's) uit verschillende bronnen (*P. rubens*, *Aspergillus nidulans* en *Saccharomyces cerevisiae*) werden synthetische promotors geconstrueerd. BGC's kunnen een expressie vereisen die voor elk gen afzonderlijk is afgestemd, en dit wordt ondersteund met de elementen die uit dit systeem zijn gekozen. De kracht van de transcriptionele controle toonde schaalbaarheid door verschillende modulaire elementen van de expressie te veranderen. De transcriptionele controleapparaten werden gekarakteriseerd met betrekking tot drie van hun hoofdcomponenten: expressie van de STF's, stroomopwaartse activerende sequenties en de affiniteit van het DNA-bindende domein van de TF voor het stroomopwaartse activerende domein. De regulerende systemen werden geëvalueerd in microbioreactor-culturen met behulp van een rode fluorescerende reporter (DsRed-T1-SKL) die het gen van belang vertegenwoordigt. Door de STF- en QUAS-elementen te integreren in de natieve CP's van het penicilline BGC in *P. rubens*, kwam de productie onder controle van de STF en was de penicilline-titer afhankelijk van het aantal geïntegreerde QUAS-elementen.

Hoofdstuk 3 beschrijft de constructie van een niet-integratief, op schimmel vector gebaseerd, en *in vivo* tot expressie gebracht CRISPR/Cas9 bezorgsysteem dat te gebruiken is in filamenteuze schimmels. De uit schimmel afkomstige promotors werden getest voor de *in vivo* expressie van CRISPR componenten in een geoptimaliseerde *P. rubens* stam waarin non-homologous end-joining (NHEJ) en DNA-reparatie ontbreken. Cas9 eiwit expressie in *P. rubens* werd vervolgens gevisualiseerd doormiddel van een GFP-NLS tag. Het Cas9-eGFP-NLS transcriptie

onderdeel werd afgeleverd via een modulaire shuttle vector (beschreven in **Hoofdstuk 5**) die compatibel is met schimmels. De desbetreffende CRISPR/Cas9 vectoren werden getest voor het bewerken van het genoom via een op fluorescentie gebaseerd knock-out experiment, waarbij een schimmel stam werd gebruikt die continu DsRed tot expressie brengt (vermeld in **Hoofdstuk 2**). Identificatie van een natuurlijk aanwezige polymerase III tRNA[Serine] promotor, een gpdA polymerase II promotor, en een (HH en DHV) zelfklievende ribozym sequentie, maken de *in vivo* bezorging van sgRNA in *P. rubens* mogelijk. We hebben het HH-sgRNA-HDV expressie systeem gekozen voor toekomstig werk, aangezien eerdere resultaten hebben laten zien dat dit systeem in meerdere schimmelsoorten werkt. De bewezen CRISPR/Cas9 schimmel shuttle vector (pLM-AMA-15) bevat phleomycine en terbinafine resistentie markers, een Cas9 expressie cassette, en een sgRNA acceptor site, wat een snelle constructie van HH-sgRNA-HDV expressie eenheden, en het opbouwen van een library, faciliteert. De CRISPR vector en DNA, dat korte homologe flankerende regionen (106 bp) bevat, werd gebruikt voor een markerloze knock-out van een N-terminal pseudo condensatie domein van het *pcbAB* gen (Pc21g21390, ACV synthetase), wat cruciaal bleek te zijn voor de functionaliteit van het eiwit. Aangezien de vector backbone autonome replicatie ondersteunt in verscheidene filamenteuze schimmels, gecombineerd met het feit dat we bewezen functionerende genetische elementen hebben gebruikt om de CRISPR componenten af te leveren, verwachten we dat dit CRISPR/Cas9 systeem ook te gebruiken is in andere schimmelsoorten.

Hoofdstuk 4 beschrijft de ontwikkeling en toepassing van een op CRISPR-gebaseerde transcriptionele activatie tool (CRISPRa/dCas9-VPR) die van toepassing is op transcriptionele inactieve genen in *P. rubens.* De dCas9-VPR en de zelfklievende sgRNA's werden getransporteerd in *P. Rubens,* via een schimmel replicatie vector, die compatibel is met verscheidene filamenteuze schimmelstammen. Bovendien maken de modulaire sgRNA insertie regionen het mogelijk om snel een library te construeren (zie **Hoofdstuk 3**). De CRISPRa/Cas9 vector (pLM-AMA-18) werd getest voor transcriptionele activatie van het DsRed

gen (geïntegreerd in het penicilline-locus van *P. rubens*), dat door de penDE core promotor aangedreven wordt. Deze promotor (al eerder genoemd en getest in **Hoofdstuk 2**) blijkt zelf de fluorescente reporter onvoldoende tot expressie te brengen, en is dus afhankelijk van de aanwezigheid van een QUAS-bindende STF. De gebouwde CRISPR/dCas9-VPR vectoren werden zo ontworpen dat de sgRNA's die worden ingezet, in de buurt van de transcriptie start regio van het doel gen binden. Wanneer vervolgens actief CRISPR/dCas9-VPR getransformeerd werd in *P. rubens*, kon met behulp van microscopie worden aangetoond dat bij 3 van de 6 geteste spacers de fluorescentie duidelijk toeneemt in vergelijking met een controle die geen sgRNA tot expressie brengt. Om aan te tonen dat de CRISPRa vector een geheel inactieve BGC kan opreguleren, en daarmee de productie van een metaboliet kan induceren, werd de macR TF van het van nature aanwezige en transcriptioneel inactieve Macrophorin biosynthese cluster gekozen als test doelwit. Van de 20 geteste spacers, die de complete promotor van macR bestrijken, werd bij 2 spacers transcriptionele activatie van het Macrophorin BGC geobserveerd, wat resulteerde in de productie van secundair metaboliet. Alles tezamen kan het geteste CRISPRa systeem gebruikt worden voor een snelle en gemakkelijke activatie van transcriptioneel inactieve genen, zonder dat daarbij genetische manipulatie van de host nodig is.

Hoofdstuk 5 beschrijft de constructie van een genetische toolkit voor synthetisch biologische toepassingen voor filamenteuze schimmels. Deze Fungal Toolkit (FTK) voor modulair klonen bevat 96 kant-en-klare, gekarakteriseerde genetische elementen die in verschillende combinaties kunnen worden geassembleerd op basis van Golden Gate klonering. De collectie bevat natuurlijke en synthetische promotors (constitutief en induceerbaar), terminators, fluorescerende reporters en selectiemarkers. Verder zijn er regulerende en DNA-bindende domeinen van transcriptionele regulatoren en componenten voor de constructie van STF's of het implementeren van verschillende op CRISPRa/i gebaseerde technologieën. Genetische onderdelen kunnen worden geassembleerd tot complexe multipartiete assemblages en worden geleverd door middel van genomische integratie of

worden uitgedrukt vanuit een op AMA1-sequentie gebaseerde, schimmel-replicerende shuttle-vector. De ingangsvectoren die coderen voor P*40s*, d*SpCas9*, *eGFP-NLS* en T*tif35* werden geassembleerd op een schimmel shuttle-vector (pLM-AMA002) die compatibel is met modulair-kloneren. De geconstrueerde transcriptie-eenheid werd afgeleverd aan *P. rubens* om de expressie te valideren met behulp van een Cas9-eiwit zonder nuclease activiteit, maar met een groen fluorescerend eiwitlabel met behulp van fluorescentiemicroscopie. Verder werd de Fungal Modular Cloning Toolkit toegepast om 22 synthetisch transcriptionele controleapparaten te bouwen (**Hoofdstuk 2**), transcriptie-eenheden voor CRISPR/Cas9-genoombewerking (**Hoofdstuk 3**) en voor CRISPRa-toepassingen (**Hoofdstuk 4)**. Met deze toolkit kunnen synthetische transcriptie-eenheden met gekarakteriseerde promotors, fusie-eiwitten of synthetische transcriptionele regulatie-apparaten sneller worden geassembleerd op een gestandaardiseerde en modulaire manier voor nieuwe schimmelcelfabrieken.

Alles bijeengenomen, hebben we een schimmelexpressiesysteem ontwikkeld, waarbij genen van een BGC orthogonaal tot expressie kunnen worden gebracht onder controle van een STF. We hebben een schimmelshuttle-vector geconstrueerd die een snelle bibliotheekconstructie faciliteert voor CRISPR/Cas9-gemedieerde genoombewerking. We hebben een nieuw op CRISPR gebaseerd transcriptioneel activeringsplatform (CRISPRa/Cas9-VPR) opgezet, dat transcriptionele activering van transcriptioneel- stille BGC’s in *P. rubens* mogelijk maakte. Aangezien synthetische biologie de ontwikkeling van nieuwe manieren voor transcriptionele regulatie mogelijk maakt, is het vaststellen van deze hulpmiddelen en methoden voor filamenteuze schimmels - organismen die van nature sterke producenten zijn van secundaire metabolieten - van cruciaal belang om hun biosynthetische potentieel volledig te benutten. Alle tools die in dit proefschrift zijn ontwikkeld, hebben tot doel het verborgen potentieel van cryptische BGC voor schimmels te ontgrendelen en de synthetische biologie voor filamenteuze schimmels te versnellen, iets dat de academische en industriële fungi gemeenschap wereldwijd zal bedienen.

Összefoglalás

Másodlagos anyagcseretermék (MA-ek) csoportját különböző molekulák alkotják, amelyeket a természetben növények, baktériumok es gombák szintetizálnak. A MA-ek bioszintézise során az elsődleges metabolizmusból származó metabolikus intermedierek, mint építő elemek kerülnek felhasználásra a MA-ek vázának szintetizálása során, amelyeknek vázát általánosságban nem-riboszómális peptid-szintetázok (NRPS-ek), poliketid-szintetázok (PKS-ek), vagy ezen enzimek kombinációi állítanak elő. Az elérhető szekvenált gomba-genomok növekvő számának köszönhetően MA-ek szintéziséért felelős bioszintetikus génklasztereinek (BGC) kész palettája került felfedezésre, melyek többségének az általuk szintetizált MA-e jelenleg ismeretlen. A legtöbb gomba MA BGC azonban transzkripcionálisan néma laboratóriumi növekedési körülmények között, és mivel a klaszterek transzkripciós aktiválását kiváltó jel vagy inger gyakran ismeretlen, ezáltal az ezek a BGC-ek „kriptikusak" teszi. A *Penicillium rubens* (korábban *P. chrysogenum*) 33 olyan BGC-t tartalmaz melyek potenzialisan egy MA vázának szintéziséért felelősek (10 NRPS, 20 PKS, 2 hibrid NRPS–PKS és 1 dimetil-allil-triptofán szintáz), ezeknek a MS BGC-nek –annak ellenére, hogy *P. rubens* tagja az egyik legtöbbet vizsgált nemzetség tagja 354 fajjal– több mint felének a szintézis terméke ismeretlen. Figyelembe véve a gombavilág óriási sokféleségét és azt az elképzelést, hogy a BGC-k széles körben elterjedtek, nyilvánvaló vált, hogy az eddig azonosított MA-ek csak a jéghegy csúcsát képzik. A szintetikus biológia megjelenésével új módszerek és eszközök váltak elérhetővé a gombakutatás területén, melyből számos alkalmazható a kritikus BGC-ek transzkripciós aktiválára.

Az 1. Fejezet áttekintést ad az MA BGC-k szabályozásáról, és összehasonlítja a jelenleg elérhető modernebb és hagyományos módszereket, melyek alkalmazhatóak BGC-ek transzkripciós aktiválásra és újra-programozására. Ezen módszerek közé tartoznak a szintetikus transzkripciós faktorok (STF-ek) alkalmazása, a mesterséges transzkripciós egységek összeállítása, a teljes génklaszter-refaktorálás, a gomba shuttle-vektorok és a heterológ expressziót szolgáló platform-

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törzsek alkalmazása.

A 2. Fejezet leírja egy ortogonális, szintetikus transzkripcionális regulációs eszköz létrehozását *P. rubens*-ben, szintetikus transzkripciós faktorok és szintetikus promóterek kombinációjával. A STF két transzkripciós faktor fúziójából tevődik össze: a DNS-kötő domén *Neurospora crassa*-ból a kínasav metabolizációért felelős transzkripciós faktorból (qa-1F-DBD), míg a transzkripcionális aktivációs domént a herpes simplex virusból (VP16-AD) került hasznosításra. A STF egy zöld fluoreszcens proteinnel lett megjelölve, valamint az SV40 sejtmagi lokalizációs szekvenciával. Mivel a DNS szekvencia ismert, amihez a qa-1F-DBD kötődni képes a *N. crassa*-ban (QUAS), ezeknek a rövid szekvenciáknak integrációja megcélzott promóterekbe növelheti azok gén expressziós képességét. QUAS szekvenciák és minimális promóterek (MP) (különböző forrásokból, úgy mint *P. rubens*, *Aspergillus nidulans*, és *Saccharomyces cerevesiae*) fúziójából szintetikus promóterek kerültek létrehozásra. A gének expressziója a BGC-ban megkívánhat ezen gének individuális regulációját, ami egy lehetségessé válik az épített rendszer elemeinek megfelelő felhasználásával.

Az transzkripcionális regulációs eszközök erőssége skálázhatóságot mutatott az alkalmazott moduláris elemek használata alatt, a választott moduloktól függően. A létrehozott regulációs eszközök karakterizálásra kerültek három fő nézőpont szerint: a STF expressziójának erősségének hatása, QUAS szekvenciák számának hatása, és a kiválasztott MP erőssége szerint. A *P. rubens* genomba integrált regulációs eszközök mikro-bio-reaktor (BioLector) kultivációk segitségével kerültek elemezésre, ami során a STF által aktivált vörös fluoreszcens protein expressziója lett mérve, ami a kísérlet során jelképezni hivatott egy a jövőbeni célzott gént. Miután QUAS elemek kerültek integrálásra a penicillin BGC eredeti promótereibe *P. ruben*-ben, a létrehozott törzsekben a penicillin-termelés detektálhatóan QUAS elemeinek számától függő volt.

A **3. Fejezetben** egy DNS-vektor-alapú, gomba-kompatililis, autonóm replikálódó, nem-integrálódó, *in vivo* CRISPR/Cas9 (CRISPR-asszociált protein 9) exp-

resszáló rendszer létrehozását írja le fonalas gombák számára. A CRISPR komponensek *in vivo* expressziójára alkalmazható gomba promóterek egy non-homológ rekombinációban deficiens *P. rubens* törszben lettek evaluálva. A Cas9 fehérje expressziója vizualizálásra került egy zöld fluoreszcens fehérje (GFP) fúziójának köszönhetően. A létrehozott Cas9-eGFP-NLS transzkripciós egység egy „moduláris klónozással" (Modular Cloning) kompatibilis gomba shuttle DNS vektor (látsd **5. Fejezet**) segítségével került megépítésre. A megépített CRISPR-Cas9 elemeket hordozó vektor evaluálása egy vörös fluoreszcens protein kiütésével történt egy olyan *P. rubens* törzsben (látsd **3. Fejezet**), amely azt konstitutívan expresszálja. A *P. rubens*-ből származó RNS-polimeráz III tRNA[Szerin] promóter valamint a pgdA RNS-polimeráz II promóter egy önhasító ribozim RNA szekvencia párral (HH és HDV) kombinálva sikeresnek bizonyultak *in vivo* CRISPR vezető RNS (sgRNA) szolgáltatására *P. rubens*-ben. Mivel HH-sgRNA-HDV szekvenciák több különböző gomba törzsben is sikeresen került alkalmazása, munkánkat ezzel a rendszerrel folytattuk. Az így létrehozott autonóm replikálódó CRISPR/Cas9 és sgRNA szolgáltató vektor DNS (pLML-AMA-15) tartalmaz egy fleomicin és egy terbinafin rezisztencia markert, Cas9 expressziós transzkripciós kazettát, valamint egy sgRNA klónozási befogadó szekvenciát, amiből az utóbbi gyors HH-sgRNA-HDV klónozást és könyvtárkészítést hivatott elősegíteni. A vektor DNS egy szelekciós-marker-mentes donor DNS-sel együtt került transzformálásra, ami során a donor DNS rövid (106 bp) homológ szekvenciák segítségével a pcbAB gén (Pc21g21390, ACV szintetáz) N-terminális pszeudokondenzációs doménjének a kiütését célozta meg. Az eltávolított pszeudokondenzációs domén egy eddig nem ismert, kritikus funkciót fedett fel az enzim működésének szempontjából. Mivel a vektor DNS autonóm replikációt biztosit több fonalas gombában is, és mivel ismert és standardizált genetikus elemeket használtunk a CRISPR komponensek expressziójára, elvárhatónak tűnik, hogy a létrehozott rendszer átvihető más gomba törzsekbe is.

A **4. Fejezet** egy CRISPR-alapú transzkripciós aktivátor (CRISPRa/dCas9-VPR) fejlesztését transzkripcionálisan néma gének számára *P. rubens*-ben. A dCas9-

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VPR és az önhasított sgRNA kifejezése egy gombában autonóm replikálódó DNS vektoron kerültek kifejezésre, mely kompatibilis több fonalas gomba fajjal is, és a vektor moduláris felépítésének köszönhetően (lásd **3. Fejezet**) gyors vektor könyvtárépítést tesz lehetővé. A CRISPRa/Cas9 vektor (pLM-AMA-18) képessége transzkripcionális aktiválásra egy minimális promóter által kifejezett vörös fluoreszcens protein (DsRed) segítségével lett evaluálva, mely előzőlegesen került integrálásra a törzs penicillin lókuszába. Az alkalmazott minimális promóter nem képes önmagában a DsRed expressziójára (lásd **2. Fejezet**), ellenben függ a promóterbe integrált STF-kötő QUAS szekvenciák számától. A létrehozott CRISPR/dCas9-VPR vektorok által kifejezett sgRNA-k a megcélzott génnek a transzkripciós iniciációs pontjához lettek tervezve. Amikor ezek a vektorok transzformálásra kerültek *P. rubens*-be, fluoreszcens mikroszkópia segítségével 3 / 6 kipróbált sgRNA mutatott aktivitást a sgRNA-mentes kontrolhoz képest. Hogy reprezentáljuk a CRISPRa vektor képességét néma, teljes BGC-ek aktiválására és MA-ek termelésének indukálására, megcéloztuk a macR transzkripciós faktort az endogén, transzkripcionálisan néma macrophorin bioszintézis gén klaszterből *P. rubens*-ben. A megvizsgált 20 sgRNA közzül (mely az egész macR promótert hivatott lefedni), 2 sgRNA esetében a génklaszter transzkipcionális aktiválása valamint indukált MA termelés volt megfigyelhető. A létrehozott CRISPRa rendszer gyors és egyszerű módszert nyújt transzkripcionális aktiválásra, a gazda génmanipuláció nélkül.

Az **5. Fejezet** egy genetikai eszköztár felépítését irja le szintetikus biológiai alapú eszközök felépítéséhez fonalasgombákban. Ez az gomba alapú eszköztár (Fungal Toolkit - FTK) tartalmaz 96 moduláris klónozást támogató, használatra kész genetikus elemet, melyeket különböző kombinációkban kombinálhatunk össze a Golden Gate-alapú moduláris klónozási módszer segítségével. A gyűjtemény természetes és szintetikus promótereket (konstitutív és indukálható), terminátorokat, fluoreszcens riportereket, és szelekciós markereket. Továbbá megtalálhatóak benne transzkripciós faktorok regulációs és DNA-kötő doménjei, komponensek STF konstrukcióhoz, valamint CRISPRa/i teknologiai elemei. A

genetikai elemek összeállíthatóak egy komplex, multi-gén konstrukcióvá, amik genomi integráció vagy egy gombákban replikálódó AMA1-alapú vektor segítségével. A kezdeti építő elemek, melyek egy teljes transzkripciós fejeznek ki (P40s, dSpCas9, eGFP-NLS and Ttif35) összeszerelésre kerültek egy moduláris klónozással kompatibilis gomba shuttle vektoron (pLM-AMA002). A létrehozott transzkripciós egység ennek segítségével kifejezésre került *P. rubens*-ben, és zöld fluoreszcens protein és fluoreszcens mikroszkóp segítségével validálásra került a nukleáz-halott Cas9 fehérje kifejezése. Az FTK eszköztár segítségével 22 szintetikus transzkripcionális regulációs eszköz került létrehozásra (**2. Fejezet**), valamint transzkripciós egységek CRISPR/Cas9 genom szektesztéséhez (**3. Fejezet**), és CRISPRa alkalmazására (**4. Fejezet**). Az eszköztár segítségével szintetikus transzkripciós egységek hozhatóak létre, gyorsan, standardizált és moduláris módon, így összeszerelhetőek különböző fúziós fehérjék és szintetikus transzkripciós regulációs eszközök, ezzel is támogatva újszerű gomba-alapú szintetikus termelő törzsek létrehozását.

Mindent összevetve, kifejlesztettünk egy újszerű expressziós rendszert fonalas gombák számára, amiben az expresszált gének ortogonális módon kifejezhetőek egy szintetikus transzkripciós faktor irányitása alatt. Létre hoztunk egy gomba shuttle vektort, ami gyors könyvtárépítést támogat CRISPR/Cas9-alapú génszerkesztés céljából. Létrehoztunk egy CRISPR-alapú transzkripcionális aktivációs rendszert (CRISPRa/Cas9-VPR), ami segítségével néma géneket és gén klasztereket aktiváltunk *P. rubens*-ben.

Appendix

Acknowledgements
About the author
List of publications

Acknowledgements

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Curriculum vitae

László Mózsik was born on April 7th 1991, in the city of Budapest, Hungary. He graduated as a Biochemical Engineer at the University of Debrecen in 2016, where during his Master's studies he specialized in gene- and enzyme technology. During his studies, he had the opportunity to participate in an Erasmus+ mobility program, and perform his Master's thesis project at the University of Groningen at the department of Molecular Microbiology under the supervision of Prof. Arnold J.M. Driessen and Yvonne Nygård. Given his interest in fungal synthetic biology, in his Master's thesis he started to work on synthetic transcription factors for transcriptional regulation in *P. rubens*. From 2016 till 2021 László was a PhD student at the department of Molecular Microbiology at the University of Groningen under the supervision of Prof. Arnold J. M. Driessen and Prof. Roel A. L. Bovenberg in the framework of the ALERT-COFUND Marie Skłodowska-Curie actions program. He developed novel synthetic biology tools for filamentous fungi, with the aim of transcriptional regulation and activation of transcriptionally silent biosynthetic gene clusters. The results of this work are described in this thesis.

List of publications

1. Transcriptional Activation of Biosynthetic Gene Clusters in Filamentous Fungi. **Mózsik L**, Iacovelli R, Bovenberg R.A.L and Driessen A.J.M* *Front. Bioeng. Biotechnol.*, Sec. Synthetic Biology (2022). https://doi.org/10.3389/fbioe.2022.901037

2. Modular Synthetic Biology Toolkit for Filamentous Fungi. **Mózsik, L.**†, Pohl, C.†, Meyer V., Bovenberg R.A.L., Nygård Y., Driessen A.J.M.* *ACS Synth. Biol.* 2850–2861 (2021) https://doi.org/10.1021/acssynbio.1c00260

3. CRISPR-Based Transcriptional Activation Tool for Silent Genes in Filamentous Fungi. **Mózsik, L.**†, Hoekzema, M.†, A.W. de Kok, N., Bovenberg, R.A.L., Nygård Y. and Driessen, A.J.M.* *Sci. Rep.* 11, 1118 (2021) https://doi.org/10.1038/s41598-020-80864-3

4. Identification of a conserved N-terminal domain in the first module of ACV synthetases. Iacovelli, R., **Mózsik L.**, Bovenberg, R.A.L., Driessen, A.J.M.* *MicrobiologyOpen*, e1145 (2021) https://doi.org/10.1002/mbo3.1145

5. *Penicillium rubens* platform strain for secondary metabolite production. Pohl, C.†, Polli, F.†, Schütze, T., Viggiano, A., **Mózsik, L.**, Jung, S., de Vries, M., Bovenberg, R.A.L., Meyer V. and Driessen, A.J.M.* *Sci. Rep.* 10,630 (2020) https://doi.org/10.1038/s41598-020-64893-6

6. Synthetic control devices for gene regulation in *Penicillium chrysogenum* **Mózsik, L.**, Büttel, Z., Bovenberg, R.A.L., Driessen, A.J.M. and Nygård Y.* *Microb. Cell. Fact* 18, 203 (2019) https://doi.org/10.1186/s12934-019-1253-3

7. Genome Editing in *Penicillium chrysogenum* Using Cas9 Ribonucleoprotein Particles. Pohl C., **Mózsik L.**, Driessen A.J.M., Bovenberg R.A.L.*, Nygård Y. *Synth. Bio.: Meth. in Mol. Bio.* (2018) https://doi.org/10.1007/978-1-4939-7795-6_12

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