Adding tools to the box: facilitating host strain engineering of Penicillium chrysogenum for the production of heterologous secondary metabolites

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CHAPTER

1

Genome editing and metabolic engineering of filamentous fungi for biosynthesis of natural products

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The kingdom of fungi is perceived as an unexplored reservoir for novel bioactive compounds that are mainly categorized as secondary metabolites. These molecules can be integrated in screening libraries for agricultural, pharmaceutical or food and feed applications. However, expression of genes required for production of these lead compounds in a heterologous organism suitable for large-scale production can be challenging. The scope of this PhD project was the construction of a secondary metabolite (SM) devoid *Penicillium chrysogenum* strain that can be used as generic host for insertion of interesting secondary metabolite gene clusters. To build this strain in a reasonable time, a more reliable genome editing tool was required. **Chapter 1** introduces the transformation of filamentous fungi and approaches for genome editing with a focus on the RNA-guided DNA endonuclease Cas9. Furthermore, design principles for the expression of heterologous gene clusters will be discussed.

Introduction

Due to a dramatic increase in antibiotic resistance in the last 20 years, development and identification of new antibiotics remains an urgent and unmet need. However, a decreasing interest of pharmaceutical companies to invest in antibiotics research became obvious during the last 15 years[1,2]. Consequently, a joint EU-US taskforce for evaluating how to stimulate research into antibiotics was initiated in 2011[3]. Besides financial benefits such as funding expensive phase III clinical trials partly by the public sector and lowering the requirements of the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for market entries[1], also research stimulation was included in the recommendations[3]. Amongst others, within the EU, this was realised within the 7th framework programme by funding projects that execute research in the field of antibiotic resistance mechanisms and identification of novel natural products.

Such a project of the 7th framework programme was Quantfung (project reference 607332), a Multi-Partner Initial Training Network (ITN) that started in October 2013. The objective of Quantfung was to apply combinations of fungal biology and synthetic biology for mining novel secondary metabolite biosynthetic gene clusters (BGCs) from filamentous fungi. Secondary metabolites produced by BGCs could not only be useful candidates for screening as antimicrobials but could also fit other applications in medicine, agriculuture and food and feed supplement applications[4–6].

Fungi as untapped source of natural products

The kingdom of fungi is considered to consist of 1.5 million species[7]. The produced specialized molecules of fungi may be an immensely rich source for new, naturally produced bioactive molecules[8,9] that cannot be obtained through classical chemical synthesis[10]. These molecules are not required per se for survival of the producing organism but are beneficial for host infection, act as defense against foragers or provide an advantage in resource competition[11,12] with other species thriving in the same environment. Fungal genomes usually contain genes for expression of different secondary metabolites (SMs) with mostly unknown function[13,14]. Moreover, some SMs may have a beneficial effect as pigments that protect from UV-induced mutations in spores[15].

Fungal SMs are initially formed through condensation reactions of required primary building blocks which are assembled by large mega-enzymes such as polyketide synthases[16] (PKS), utilizing coenzyme-A coupled fatty-acid building blocks, non-ribosomal-peptide synthases[17] (NRPS) requiring amino acids or terpence cyclases[22] (TC), which utilize dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) as precursor molecules. Since the enzymes involved in these reactions have a large, multi-domain structure with several conserved domains and sequence motifs, their presence in a genome can be detected by computational tools like AntiSmash[19,20] or SMURF[21]. These bioinformatic methods serve as an anchor point for the presence of associated genes that form a biosynthetic gene cluster, containing all the necessary enzymes to synthetize a SM. Interesting possibilities arise

from these metabolite mining pipelines, e.g. about 1000 fungal genomes are going to be sequenced in the coming years by the Joint Genome Institute (JGI) 1000 fungal genomes project[22] and several species of the genus *Penicillium*[14] and *Aspergillus*[23] were sequenced recently, offering the possibility to track the occurrence of BGCs across different species and isolates, which may reveal missing genes as already demonstrated for *Aspergillus fumigatus*[24]. However, it should be noted that the bioprospecting of cryptic fungal biosynthetic gene clusters (BGCs) should follow purpose-led rationales which can either be directed towards understanding the biological function of a SM in the producing organism or to discover SMs that suit a targeted application, i.e. in crop protection, food, feed or healthcare. For each application, it is mandatory to have a clear plan which molecular structure or physicochemical properties (molecule radius, molecular weight, charged groups) is required to narrow down the search space for BGC-derived products that are shortlisted for mining. This can be achieved by knowing the interacting molecules of an enzyme (i.e the substrate) or by performing protein-ligand docking simulations[25] to obtain a lead structure which can be used as an initial scaffold that SM molecular structures can be compared to. Alternatively, structurally resolved SMs can always be included in a compound screening library that is tested for a specific effect for a defined application.

Design principles for overexpression of biosynthetic gene clusters

In most cases, production of some SMs is highly dependent on the received stimulus and will be absent if the fungus does not require the product[12,26]. Consequently, most fungal isolates produce only a limited set of their SMs under laboratory conditions, necessitating the use of alternative stimuly to awaken biosynthetic gene clusters. Typical are screenings varying the used source of nitrogen[27] or carbohydrate as well as stress conditions covering the depletion of enzyme cofactors such as iron[28]. Combining this knowledge with information gained from ongoing sequencing and annotating efforts of an increasing number of fungal genomes, will fill gaps in the "fungal secondary metabolome tree" (**Figure 1**) and ultimately allow identification of novel SM BGCs. The decisionmaking about which uncharacterized BGC to study can also be based on clustering BGC core genes together with SM structure and is remarkably useful to map the produced SM to the BGC core gene to identify and prioritize distinct or related BGCs for heterologous expression[29]. A systematic archiving of information about producing organisms and the BGC of a SM in a unified format is realized by the the Minimum Information about a Biosynthetic Gene Cluster (MIBiG) specification database[30].

Further, having access to multiple genomes containing the same BGC might also enable scientists to perform a more rational selection of silent but functional BGC candidates that can produce secondary metabolites when expressed in a heterologous host (**Figure 1**). A recent study[24] in *A. fumigatus* identified several gene clusters with point mutations in the core genes of a BGC and the encoded core BGCs of industrially used *Penicillium chrysogenum* strains[31] also contain point mutations that would hamper successful compound production when resorting to this strains as origin of the respective core enzyme.

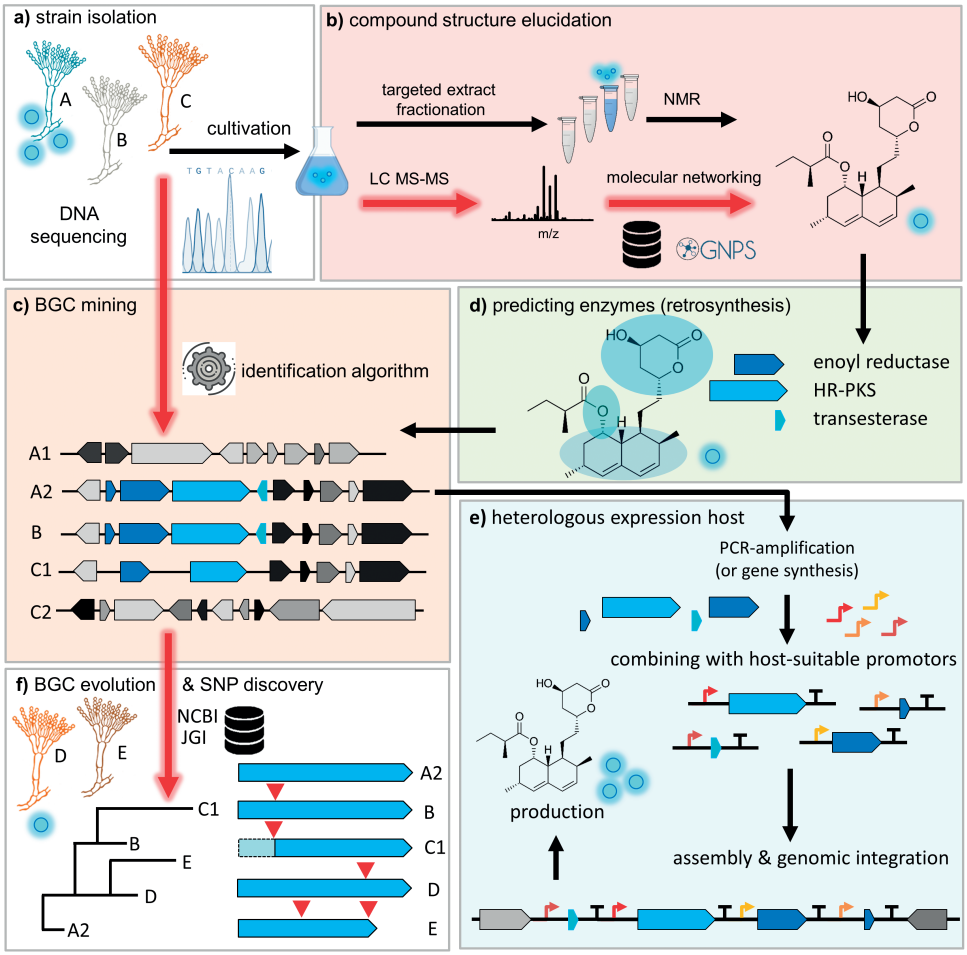

Figure 1. Schematic identification and heterologous expression of a BGC producing a secondary metabolite of interest. Red arrows present efforts that can lead to better understanding of BGC diversity and evolution. **a)** Strain isolation as an initial start to compound discovery should be accompanied by genome sequencing in order to obtain an overview of the genomic landscape facilitating BGC identification. **b)** Molecular structures can either be identified (*de novo*) using fractionation and NMR or by extensive MS/MS analysis and comparison to sample libraries using molecular networking to obtain coarse structural fingerprints. **c)** BGC mining of sequenced genomes is leading to a predicted cluster of enzymes showing typical protein domains for natural product synthesis and various BGC core genes. **d)** Predicting enzymes involved in synthesis of a compound can assist in delineating the required enzymes for production of an identified compound. **e)** Selected genes required for production of the target molecule are refractored to be compatible with the heterologous expression host by exchanging the promoter to a host-compatible version, adjusting the codon usage and removing introns, when necessary. **f)** Increasing numbers of sequenced strains can be searched for similar BGCs to gain more insights into potential mutations and evolutionary events leading to the failure of compound production in closely related strains

These observations underpin that BGC mining can be a high-risk endeavour. Multiple errors can arise due to the evolutionary trajectory of a BGC, rendering it nonfunctional without the possibility to identify the root cause due to the lack of a similar cluster in another species or due to errors stemming from automated genome annotation pipelines (ORF annotation and splicing prediction errors). These errors cannot be debugged with transcriptome data when the cluster in focus is silent. Consequently, a heterologous expression strategy needs to be able to accommodate quick changes in the DNA constructs tested, preferably allowing direct integration of multiple DNA parts that can be redistributed or replaced by modified parts build with the knowledge gained after a first design-build-test-learn cycle (**Figure 1**). Yet, the current status of genetic part integration into filamentous fungi build with the paradigms of synthetic biology[32] in mind is promising, with success of integrating three parts into three distinct loci enabled by the use of targetable nucleases[33] and eight parts into a single loci (Chapter four of this thesis), however still underexeeding the recombineering of more than 14 diverse parts with short overlaps in *Sacchromyces cerevisiae* as demonstrated in the HEX system[34] for heterologous expression of fungal PKS-based BGCs. It would be worth to further examine the upper boundary of co-transformable parts in filamentous fungi to be on par with single-celled fungi (yeasts) and to increase usage of synthetic DNA fragments with adapted codon usage and removal of unwanted features of the nucleic acid sequence (i.e. restriction enzyme sites).

Typically for heterologous expression of pathways in filamentous fungi is the application of combinatorial cloning methods like GoldenBraid[35] or MoClo[36], which are based on iterative use of type-II restriction enzymes utilized for construction of expression cassettes. The desired host for heterologous expression should have a selection of characterized promoters which allows facile building and subsequent integration of multiple ORFs with a fixed or tunable expression output (**Figure 1**). The benefit of this strategy is twofold: on one hand, it reduces risk of spontaneous loop-out of introduced parts where similar sequences are used multiple times, especially when the produced SM can exert severe stress to the host organism. This genetic instability is in fact frequently exploited for forced removal of the acetamidase gene *amdS* by using the toxic metabolite 5-fluoroacetamide[37,38] but is highly unwanted in long-term cultivations or expanding culture volumes in upstream processing of cultivations without the means to inhibit product formation in place. On the other hand, application of well characterized promoters allows the fine-tuning of enzyme amounts in the cell, which might be required to achieve optimal pathway performance.

Amongst fungal model organisms like *Aspergillus nidulans*, *Neuraspora crassa*, *Trichoderma reesii*, *P. chrysogenum* is a filamentous fungus with a selection of characterized constitutive promoters[39], yet there are a few publications describing inducible systems for filamentous fungi based on the addition of small molecule-based inducers such as xylose[40], arabinose[41], starch[42], doxycycline[43] or ethanol[44]. Unfortunately, most of these systems do not fully characterize the inducible system in a comparable fashion (i.e., the assay used for output quantification varies substantially and the dynamic range of a promoter is seldomly accessed completely) for filamentous fungi in general. Future work in the domain of filamentous fungi

should therefore aim on a consensus how to judge the performance of a promoter to allow better outcome prediction of promoter activity.

Heneghan et. al[45] could still resort to repeated usage of the *amyB* promoter to express the four known and well-characterized genes *tenA, tenB, tenC* and *tenS* from *Beauveria bassiana* to heterologously produce the SM tenellin in *A. oryzae* using time-cosuming subcloning. However, the expression of larger clusters with entire refactoring of the cluster remains a challenge and will likely require more promoters to be characterized. A recently introduced tool for bacteria that allows systematic expression optimization if up to 12 genes is the *Escherichia coli* Marionette system[46], combining 12 small-molecule inducible promoters to determine optimal pathway performance without the need to repeated promoter swapping. Notably, also the choice of genomic locus for integration of expression cassettes might have an impact as demonstrated for *A. niger*[47] due to different chromatin accessibility and chromosomal condensation status. Importantly, the performance of each promoter usually holds only true for one organism and importing a promoter to another organism (i.e. when required for host onboarding of an interesting species) might give entirely different outcomes.

Rantasalo and coworkers therefore developed a generic, synthetic expression system (SES)[48] that is capable of achieving similar performance in different fungal hosts.

Upcoming research projects dealing with pathway integration and characterization into a filamentous fungal host can already resort on a solid foundation for integration and expression of heterologous genes. However, for successful compound identification, researchers are highly encouraged to take advantage of and give support to untargeted metabolomics data sharing platforms such as the Global Natural Products Social Molecular Networking (GNPS)[49] website. These databases can facilitate can facilitate identification of produced compounds through molecular networking between publicly available raw data and own samples as recently demonstrated for identification of fungisporin-related molecules in *Penicillium nordicum*[50].

Transformation of filamentous fungi

State-of-the-art genome editing in *P. chrysogenum* is done mostly via homologous recombination of large pieces of DNA that needs to contain a selectable marker[51], flanked by homologous sequences for targeted insertion into the fungal genome. Delivery of these genomic integration cassettes is achieved via different routes, amongst them breakdown of the fungal cell wall by lytic enzymes and PEG-mediated DNA-uptake into the osmotically stabilized protoplasts[52], agrobacterium-mediated delivery[53], electroporation and biolistic approaches[54]. In the following section, a more detailed description of the procedures used for preparation and transformation of fungal protoplast will be given.

In order to obtain protoplasts from filamentous fungi, the fungal cell wall[55,56] needs to be removed to obtain single cells and to allow passage of the DNA through the cell membrane (**Figure 2**). The fungal cell wall is structured highly different between fungal species yet is constituted primarily from three polymerized units: chitin, α- and ß-glucans but can also

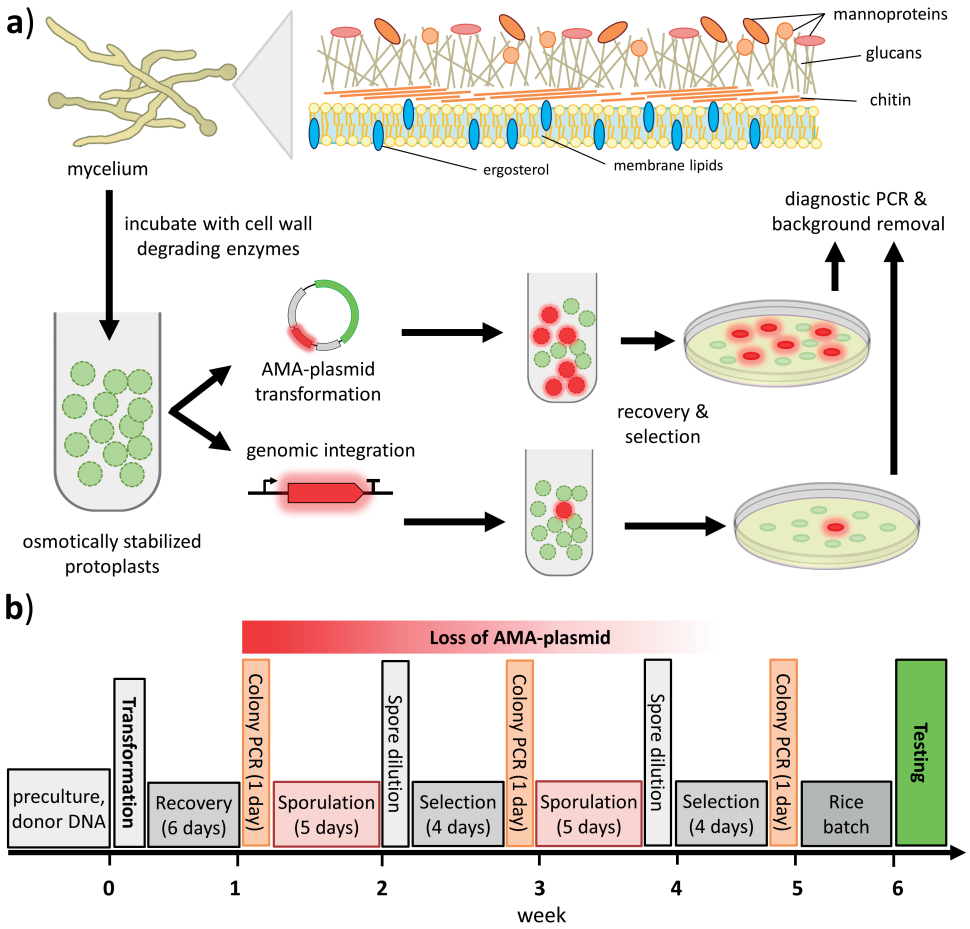

Figure 2. Transformation workflow and time demand for generating a single-site genome-edited filamentous fungi. **a)** Scheme for a protoplast-based fungal transformation. Mycelium is harvested and incubated with cell-wall lysing enzymes, allowing the uptake of a selectable donor DNA through the cell membrane in the presence of high PEG concentrations. DNA-containing protoplasts are selected on regeneration plates, sufficiently suppressing the growth of not-transformed protoplasts. Obtained colonies are subjected to diagnostic PCR and subsequent purification steps **b)** Estimated time for obtaining pure fungal clones suitable for experimental testing. Repeated spore isolation and colony PCR is required to ensure only propagation of positive clones and purge background. If AMA-plasmids do not contain an essential gene or a selectable marker, they will not be propagated via spores and are hence lost.

be decorated with arabinan, galactomannan, mannoproteins and other integrated proteins. The degradation of the cell wall is performed with a mixture of hydrolytic enzymes (Glucanex, Yatalase, Driselase) sourced from microorganism genus of *Trichoderma* or *Corynebacterium*, containing various chitinases, chitobiases and β-1,3-glucanase able to degrade fungal or plant cell walls. During protoplast formation protoplasts are maintained in a liquid medium containing osmotic stabilizers such as salts or sorbitol to avoid bursting of protoplasts.

Similarly to eukaryotic cells, a cryoprotectant preventing ice crystal formation such as polyvinylpyrrolidone 40 can be added to the osmotically stabilized protoplasts and allows storage of protoplasts for several weeks at -80°C[57].

The delivery of donor DNA into the protoplasts is performed by addition of a high concentration of polyethyleneglycol (PEG), presumably evoking a distortion of the cell membrane and forcing the negatively charged DNA in contact with the cell membrane. DNA delivery into fungal protoplasts and import of the DNA into the nucleus is achieved with sufficient efficiency as shown with the delivery of AMA-plasmids that can be maintained in fungi, reaching a much higher frequency of colonies on regeneration plates compared to a donor DNA which requires integration into the genome[58,59]. This points at strikingly different activities of DNA repair and surveillance mechanisms in filamentous fungi and *S. cerevisiae*, the benchmarking organism for homologous recombination efficiency in the fungal kingdom of life. After recovery of protoplasts on osmotically stabilized solid medium, the presence of the introduced donor DNA is verified by PCR diagnostics. Additionally, the frequent utilization of dominant selection markers (*amdS, ble, hph, ergA*) in *Penicillium* and the relative low number of successful transformed protoplasts (usually below 1%) in combination with rapid expansion of hyphae from transformants leads to frequent escape-growth of non-transformed protoplasts that require multiple rounds of purification to ensure that genetically pure strains are obtained (**Figure 2b**).

Moreover, removal of the introduced selection marker (via LoxP/Cre system or by counterselection) requires several weeks and slows down genetic engineering of filamentous fungi. As of 2014, no evidence in literature exists for a system that allows multiplexing or a faster read-out of transformation results. To summarize, the systems available for genetic engineering in *P. chrysogenum* and other fungi at this time did not allow a fast strain construction which would be favourable to speed up the heterologous expression of several genes of interest.

Genome editing approaches of interest for filamentous fungi

Genome editing tools[60] can be defined as techniques that use various means of interaction with the DNA present in an organism to specifically alter it by exchanging nucleotides. Applications for genome editing tools range from gene function studies by means of targeted gene knockouts to mutational studies, for instance to improve the catalytic active domain of an enzyme, to production host engineering in biotechnology involving multiple targets in the genome. Genome editing requires recognition of a specific stretch of nucleotides by a protein (that may act as a carrier for a complementary oligonucleotide sequence in some cases) which can perform manipulations on the DNA itself or is recruiting other DNA-active proteins that perform a specific, at best programmable task. Genome editing tools can be categorized by the number of nucleotides which are required for recognition of the target site and the usual size of the introduced modification. The genome editing tools available for filamentous fungi are selectable integration cassettes, Restriction enzyme-mediated integration (REMI)[61], the LoxP/Cre system, Zinc Finger Nucleases (ZNF), Transcription

activator-like effector nucleases (TALEN) or approaches based on clustered regularly interspaced short palindromic repeats (CRISPR)[60] (**Figure 3**). Of the named tools, the TALEN approach allows highly flexible targeting of the fused nuclease (often FokI, cleavage motif 5′-GGATG-3′) to a site specidied by 15 to 20 basepairs. The disadvantage of TALEN systems is the need to design a new protein for each targeting sequence[62], leading to a large reduction in targeting flexibility.

Whereas a plethora of tools and technologies for targeted altering of genome sequences was published in the last decade for well-established model organisms, only few proof-of-principle publications can be found for filamentous fungi on the use of the LoxP/Cre system[63–66]. The LoxP/Cre system requires the presence of loxP recombination sites inside the genome which needs to be introduced via an integration cassette if not present in the host genome. While LoxP sites allow facile removal or inversion of genetic elements such as selection markers, they can lead to loss of genome stability when present in multiple copies. This approach is succesfully exploited for generation of improved yeast strains[67] but requires substantial remodelling of the entire yeast genome, hence requiring a large amount of upfront preparation before executing an experiment.

Because the CRISPR system offers a high flexibility with little adaption effort required for new targets compared to the approaches described briefly before, the following section will focus on the underlying mechanism of the targetable nuclease Cas9.

Functional mechanism of the RNA-guided DNA endonuclease Cas9

Clustered regularly interspaced short palindromic repeats (*CRISPR)* were discovered first in *E. coli*[68] in 1987. The occurrence of CRISPR-associated-genes (Cas) in prokaryotic organism was systematically described in 2002[69] and the hypothesis that CRISPR/Cas serves as a resistance mechanism of bacteria against phage invasion was revealed in 2007 for S*taphylococcus pneumoniae*[70]. Since then, the mechanism of CRISPR/Cas systems has been elucidated.

CRISPR systems incorporate multiple short (20 40 bp) DNA fragments, termed protospacers in between short palindromic repeats in the CRISPR array. These arrays are transcribed by a type III RNA polymerase and further processed into CRISPR RNA (crRNA) by associated Cas-genes. Stabilized by a trans-acting CRISPR-RNA (tracrRNA), the crRNA recruits multiple proteins to form the Cas complex[71] or for type II CRISPR systems, the crRNA is recognized by single RNA-dependent endonuclease Cas9[72]. It was first shown in *E. coli*[73] that these RNA-loaded proteins can recognize the complementary sequences to the crRNA in the DNA of an invading phage if this sequence follows a short DNA motif, the protospacer-adjacent motif (PAM) which is –NGG for *Staphylococcus pyogenes*[74,75]. Upon recognition of the target sequence, a double strand break is induced in the DNA which can then be either repaired by the non-homologous-end-joining pathway (NHEJ) that may cause frameshift mutations or insertion/deletions (INDELS) or homology-directed repair (HDR) systems become active. The efficiency of correct integration events in filamentous fungi via HDR is increased by

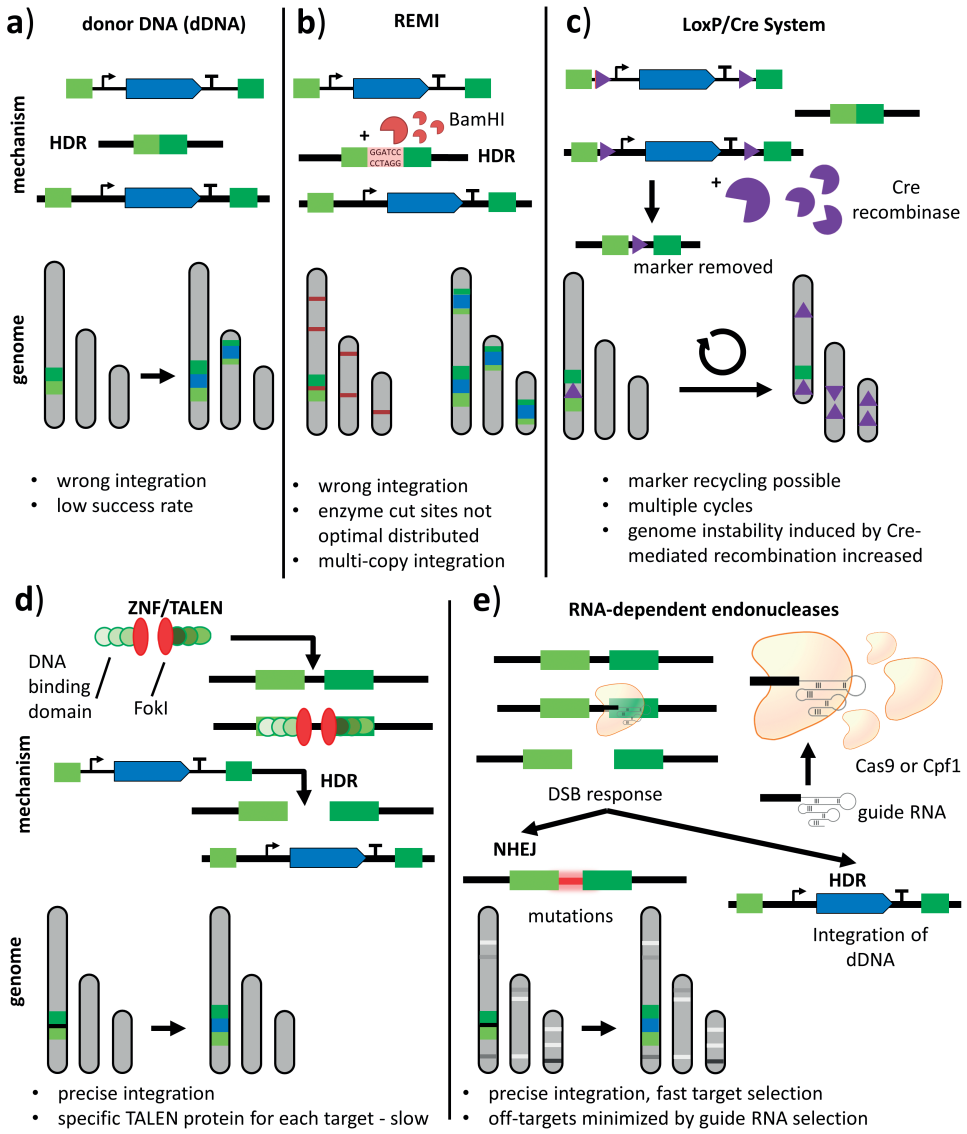

Figure 3. Schematic overview of genome editing tools and their interaction with the DNA. **a)** A selectable donor DNA is the simplest approach to introduce a desired DNA sequence, relying entirely on the organism's ability to either randomly or targeted introduce a piece of DNA to a genomic site. **b**) REMI uses restriction enzymes with few cut sites inside a genome to generate a DNA-damage stress response, thereby facilitating integration of donor DNA at specified and random locations. **c**) The LoxP/Cre system can be used to excise a selection marker or any other cargo from a donor DNA but can lead to chromosomal rearrangements when multiple LoxP sites are present. **d**) ZNF/TALEN utilize DNA binding domains that can be targeted to a specific DNA sequence, thereby bringing the FokI nuclease in close proximity to the cutting motif. Application of TALENs is slowed by the need to redesign the DNA binding domain for each target. **e**) RNA-dependent endonucleases are loaded with a small RNA molecule which specifies the DNA sequence to be cutted, greatly simplifying the efforts to target different sites in the genome.

deletion of the Ku70 protein homolog HdfA[76,77], which is required for recognizing double strand (DSB) breaks and induces the non-homologous end-joining pathway.

These findings made CRISPR/Cas9 interesting as a genome editing tool, because INDELS might cause frame-shift mutations or premature stop-codons which can lead to expression of a wrong amino acid sequence in the encoded protein or the earlier termination of transcription. Thus, by using NHEJ, gene silencing is possible whereas HDR allows a higher efficiency of introducing a donor DNA at the target locus. These donor DNA oligonucleotides can be used to introduce changes in the DNA that lead to point mutations or can even be used to introduce large genomic fragments. Furthermore, CRISPR/Cas9 does only require an sgRNA that matches the 20 nucleotides following the protospacer adjacent motif (PAM) in the genome to recognize the DNA, so no foreign DNA sequences must be introduced for genome editing. These features stimulated development of CRISPR/Cas9 based tools that further improved the Cas9 function. It was shown that a fusion of crRNA and tracrRNA, the so-called single-guide RNA (sgRNA)[78] is also accepted by Cas9, facilitating simplification of the components required for the Cas9 system to be active. Moreover, other targetable nucleases such as Cas12a/Cpf1[79,80] were identified that broadened the targetable nucleotide motifs within genomes. Soon after this, the "CRISPR-craze" started where it was demonstrated that CRISPR/Cas9 was well suited for genome editing in yeast[81], mice[82], fruit fly[83], human cell lines[84], rice[85], tobacco[86] and bacteria[87]. Also, varieties of Cas9 proteins were created such as nickases that cleave only one strand of the DNA and reduce activity of the NHEJ pathway or fusions of catalytic inactive Cas9 (dCas9) to FokI nuclease[88]. These FokI-fusions have a reduced off-target effect[89] because FokI nuclease will only cleave the DNA if the two dCas9 proteins linked to it will recognize both their targets in close proximity correctly. Also multiplexing of the system for up to three targets sites simultaneously was shown to be possible in *S. cerevisiae*[90].

However, it became obvious that careful sgRNA design and selection of CRISPR/Cas9 targets is important as the system can have off-target effects and may cleave at sequences that are almost similar to the sgRNA[91]. Systematic probing of the succeptibility of Cas9 to cause these unwanted side-effects, so-called off-target mutations, pointed towards several nucleotide positions in the protospacer motif that are contributing to a lesser extent to the specifity of Cas9[74,92]. This sparked a series of inventions to circumvent these problematic observations, including appropriate detection methods for off-targets such as Digenome-Seq[93], a plethora of tools that are able to predict off-targets into the target organisms genome[94–96] and structure-guided modifications to reduce off-target cutting[97,98]. Nonetheless, delivery of the Cas9 protein and the sgRNA in a balanced way remains important to avoid off-target effects and reduce the toxicity of Cas9 which is observed in some species.

Mostly, expression of the sgRNA and the Cas9 protein is realized via host specific vectors[81,90] which require the availability of a functional polymerase III-promoter in front of the sgRNA expression cassette to express the sgRNA in the nucleus. Recently, it has been demonstrated that also self-splicing ribozymes can be fused to sgRNA[99], thereby omitting the need for a polymerase III-promoter. If the expressed Cas9 protein is equipped with

a nuclear localization signal (NLS) it will be targeted to the nucleus after binding the sgRNA in the cytoplasm. Alternative approaches rely on stable integration of the Cas9 expression system[100,101]. Also the possibility to deliver only a pulse of Cas9 protein and sgRNA to the recipient organism has recently been shown[102] for mammalian cell lines by using cell penetrating peptides or by packaging of sgRNA and Cas9 into liposomes[103]. By using combinations of sgRNA and a donor DNA, it was also shown that targeted introduction of point mutations is possible to i.e. fix a cancer mutation in mice hepatocytes[82].

Within the ascomycete and basidiomycete fungi, CRISPR systems were established beginning in 2015, and have been demonstrated to work in 41 different fungal species until the end of 2018 (reviewed in Schuster et al.[104]). As soon as the hurdle of protoplast preparation for a fungal species is overcome and a set of working selection markers and promoters are known, a researcher can now resort to test different strategies of marker-assisted or marker-free genome editing. While Cas9 is frequently delivered encoded on a donor DNA or on a AMA1 plasmid, we could also demonstrate that protoplasts of Penicillium chrysogenum are able to take incorporate the 160 kDa Cas9 ribonucleoparticles[105] likely due to membrane perturbation during PEG-mediated DNA delivery. The applicability of protein delivery to fungal protoplasts was demonstrated before with the 38 kDa Cre recombinase [65] in *A. oryzae*. The sgRNA can either be directly delivered as a RNA molecule alongside the Cas9 expression construct or on a separate plasmid or donor DNA fragment[104]. These combinations of Cas9 and sgRNA delivery suggest that donor DNA elements and sgRNA molecules inside a fungal protoplast seem to be equally stable as in mammalian cells where an increase in Cas9 induced mutations is observed[106] only after several hours owed to the transport of DNA fragments to the nucleus, transcription of mRNA and translation of Cas9 protein. Given the challenging purification of filamentous fungi clones for testing, the greatest benefit of applying a targetable nuclease is the possibility to target multiple genetic loci, thereby greatly reducing the time to a final strain. Multiplexing of up to 3 different genomic loci was demonstrated for 11 different filamentous fungi[104] with editing efficiency of a triple modifications usually well below 40%, suggesting that the probability of taking up multiple DNA elements into a single protoplast is lower than taking up only a single fragment, hence the uptake of all required parts becomes the critical mechanism for efficient multiplexing.

To summarize, the CRISPR/Cas9 system is a tool for multiple purposes where gene deletion/gene silencing by NHEJ as well as specific introduction of point mutations or insertions via HDR were the most important applications for this research project.

Host strains and metabolic engineering for secondary metabolite production

The term generic is defined as "relating to a class or group of things; not specific" and means in general being applicable for multiple purposes. Established filamentous fungi relevant to industry derive mainly from the genus *Aspergillus, Penicillium and Trichoderma*[107–109]. These are considered excellent secretors of proteins and small metabolites such as citric acid or penicillin. However, *Trichoderma* and *Aspergillus* spp. show a strongly separated product spectrum when compared to *Penicillium*[107]. Production of a wide panel of native and recombinant proteins[110–112] and compounds of the primary metabolism such as citric acid or gluconic acid[113,114] is economically feasible using *Aspergillii spp*. such as *A. niger*, *A. awamori* and *A. terreus*. Cellulases required for e.g raw material treatment in bioethanol production are produced in Trichoderma spp[115].

In contrast, various compounds of the secondary metabolism, primarily ß-lactam antibiotics, are produced in ton-scales annually with *P. chrysogenum* and *Acremonium chrysogenum*[116]. Therefore, *P. chrysogenum* will be used for expression of SMs clusters in this PhD project because production of ß-lactams was extensively studied[117–119] and it is considered an established production host or chassis organism (as termed in synthetic biology) with a low level of excreted proteins. Industrial ß-lactam producing *P. chrysogenum* strains were traditionally developed by repeated mutagenic treatments[31,120] and subsequent selection of the high yield ~~or~~ producers or other strain characteristics (morphology, pigmentation, impurity reduction). High penicillin producing industrial strains have accumulated multiple point mutations per treatment, and this has impaired production of other, native SMs while optimizing the metabolic fluxes in the cell to higher ß-lactam titers[121]. Understanding which mutations are required for efficient production of ß-lactams will be important to generate leads for genetic engineering of *P. chrysogenum* to produce different SMs.

A generic *P. chrysogenum* host strain to produce SMs should at first have some good growth and fermentation characteristics. When grown in liquid culture, it is important that this strain grows with a dispersed morphology to allow good mixing of the fermentation broth and no oxygen and nutrient gradients which usually occur during pellet growth. A dispersed morphology is usually prefferred for protein expression[122,123] but it remainst to be seen if this always guaranties a high yield of secondary metabolites. Moreover, a reduced wall growth in culture vessels is important to ensure a homogenous broth and allows to parallel strain screening in 24-well plates or parallel bioreactor systems with a low culture volume. It is also important to select a strain of *P. chrysogenum* that has not a strong bias in amino acid metabolism. Because isopenicillin N is build up from the amino acids L-cysteine and L-valine, high penicillin-production strains could also have accumulated mutations in the pathways of these amino acids. Since production of a wide panel of secondary metabolites should be possible with a generic *P. chrysogenum* strain the amino acid-metabolism should remain flexible to produce any amino acid that could be produced in the wild type *P. chrysogenum* NRRL 1951[124].

More than 40 NRPS and PKS genes were identified in the genome-sequenced strain *P. chrysogenum* Wisc54-1255[13]. In industrial production strains, these genes are already silenced to some extent due to classical strain improvement[31]. However, it could happen that some of the still active but dormant BGCs might show activity if the metabolic fluxes are directed different than usually during penicillin production. Inactivation of the strongly expressed genes is then required to omit competition for cellular resources. The absence of other SMs will also make the downstream processing of secondary metabolites easier, avoiding co-extraction of metabolites that behave similar.

Another requirement for a generic SM producing strain is also related to the product which should be produced. Depending on the source of the SM cluster that should be expressed, the presence of cellular compartments such as microbodies or peroxisomes might be required. This can help to stabilize product intermediates and could lead to an increased yield. It will also be important to determine if a heterologous SM requires a specific transporter for secretion out of the fungal cell or even for uptake into cellular compartments. In this respect, specific knowledge about the need for transport is often lacking with SM pathways.

Once a desired BGC has been selected for heterologous production and its precursor metabolites are known, one can start to intervene in the primary metabolism to redirect the fluxes towards sufficient precursor supply as reviewed by Nielsen et. al[125]. Typically, this becomes necessary when production of the desired compound is limited by the availability of precursor molecules, such as amino acids or CoA-conjugated molecules. This can be addressed by introducing additional gene copies or alternative synthesis routes to the required building block. Since the field of metabolic engineering itself is extremely broad, it will not be attempted to provide an in-depth discussion about this topic here. The interested reader is referred to a recent review by Nielsen and Keasling[126]. Whenever possible, the engineering choices for any organism should be tested with assistance of a metabolic model (i.e. using the RAVEN toolbox[127] for *P. chrysogenum*) that can deliver predictions of the cellular flux rebalancing when interventions in the primary metabolism will become necessary. Of note here is that the increased production of cellular primary metabolites might also induce a cellular feedback that can counteract the engineering effort to increase yield by for instance negatively impacting growth rates.

Taken together, possible improvements on the host strain for production are manifold and can be implemented whenever the product titer needs to be increased further.

The benefit of SM BGC is the uniqueness of their final and intermediate products, making it important to ensure sufficient supply of precursor metabolites and identification of enzymes involved in unspecific side reactions leading to product degradation as the first optimization steps, since the produced compounds are unlikely to feed in many other metabolic pathways.

Scope of the thesis

Despite their important role in industry, genetic engineering of filamentous fungi yet lacks an extensive toolbox with the same functionality as that for *S. cerevisiae* or *E. coli*. Adaption of existing methods for genetic engineering from the latter organisms must continue in order to speed up construction of fungal strains with novel features. Furthermore, control over timing and expression level of several genes requires further research to identify promoters and tunable expression systems for better temporal control of heterologous protein expression, and to circumvent growth defects or stress-induced population heterogeneity that can cause yield loss when scaling cultivations. This thesis therefore explores new tools and principles to faster engineer *Penicillium chrysogenum* and to develop a platform strain for heterologous expression of secondary metabolites.

Chapter 1 introduces the functional mechanism of the RNA-guided DNA endonuclease Cas9 and the strategies applied to implement this targetable endonuclease in various fungal hosts. Furthermore, design principles for the overexpression of heterologous gene clusters and considerations for pre-cursor and cofactor supply will be discussed.

Chapter 2 describes the development of a Cas9 ribonucleoprotein-based transformation method for *P. chrysogenum* that improves gene deletions and construction of overexpression strains by considerably shortening the homology lenght required on the donor DNA for homologous recombination.

Chapter 3 identifies the polyketide synthetase responsible for the biosynthesis of Calbistrin in *Penicillium decumbens* through a joint effort of biosynthetic pathway mining and gene deletions using the tool developed in **Chapter 2**.

Chapter 4 utilizes the Cas9 ribonucleoprotein tool to delete genes involved in the biosynthesis of the secondary metabolites Chrysogine, Roquefortin and Fungisporin to create a clean host strain with reduced secondary metabolite payload. The obtained strain was characterized for changes of intracellular amino acid levels in glucose limited chemostats and transcriptome data were compared against a panel of penicillin-production strains. Furthermore, the polyketide synthethase PKS17 and the penicillin biosynthetic gene cluster were re-introduced to examine the performance of the newly developed platform strain for secondary metabolite production. Finally, the biosynthetic gene cluster required for the synthesis of Calbistrin identified in **Chapter 3** was transplanted into the engineered *P. chrysogenum* strain and product titers were compared to the native host.

Lastly, **Chapter 5** summarizes the development of an aldehyde-inducible promoter for Penicillium chrysogenum based on the alcR transcription factor and reports its performance for overexpression of the penicillin biosynthetic gene cluster from a polycistronic expression

construct. Furthermore, a strategy for fast selection of gain-of-function mutants in liquid culture using the *hisB* gene as an auxotrophy marker and the impact of the ornithin-decarboxylase degradation tag on the stability of DsRed is investigated.

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