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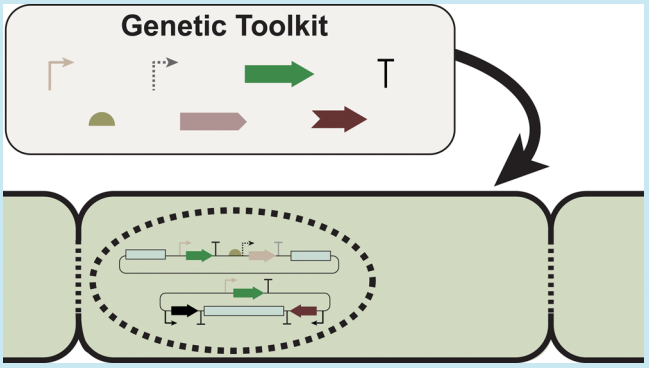
Modular Synthetic Biology Toolkit for Filamentous Fungi

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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*

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INTRODUCTION

Filamentous fungi are widely used as cell factories: organic acids, small-molecule drugs, and 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] Not only are fungi excellent workhorses for protein production because of their natural capacity for protein secretion, but also, fungal genomes 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 BGCs 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 under 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 that are responsible for natural product biosynthesis, most of which 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-tuned 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 that are already transcriptionally active, such tools can be used for the activation of transcriptionally silent BGCs and the discovery of novel natural products. 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

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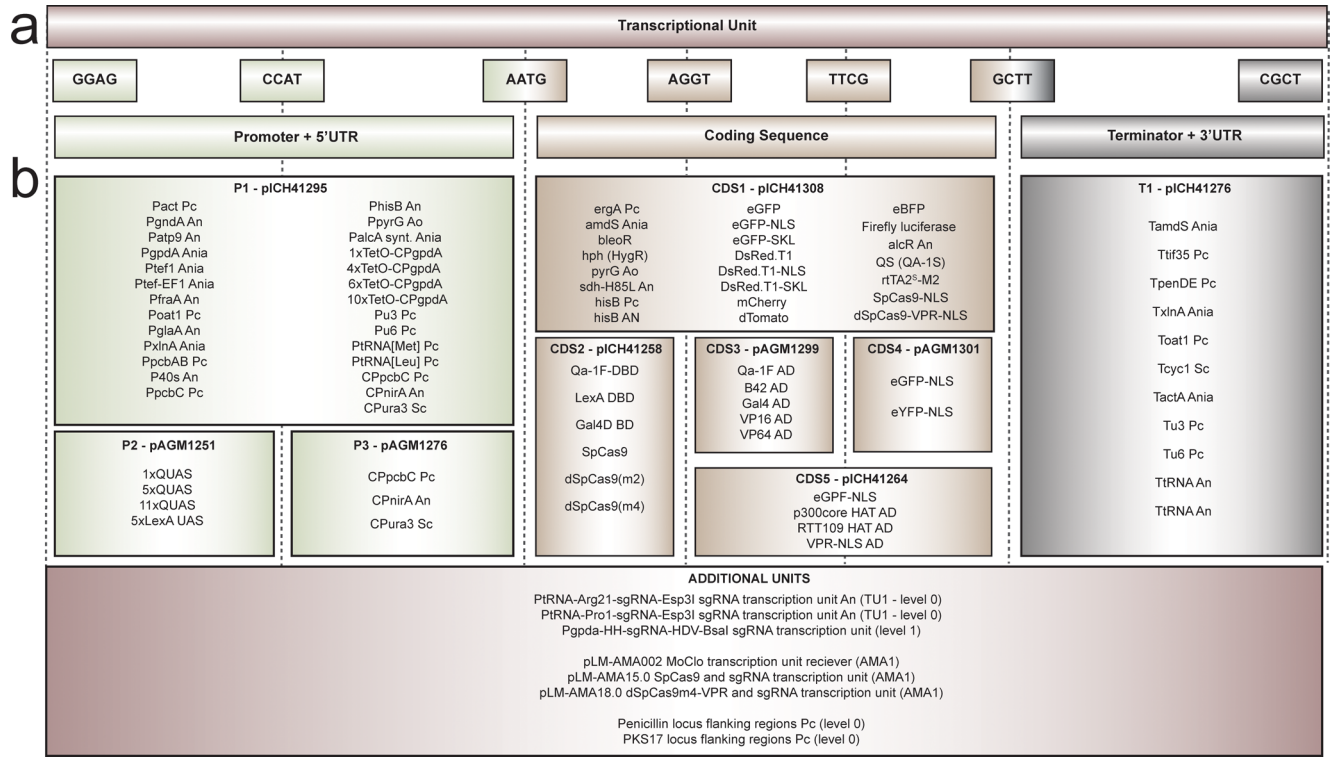


Figure 1. List of vectors in the Fungal Modular Cloning Toolkit. (a) Location of genetic parts in a transcription unit with their corresponding linker sequences. (b) List of parts of the toolkit, containing promoters (P1), UASs (P2), UAS-compatible core promoters (P3), coding sequences with various fusion possibilities (CDS1−5), terminators (T1), complete transcription units (TUs), and additional vectors (sgRNA transcription units, flanking sequences, and 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).

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 TFs or other genetic switches. By the use of 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 *Aspergillus* species,[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* species and *U. maydis*, providing inducer-based transcriptional regulation.[5,8,11] STF-based regulatory systems show transferability among a variety of different fungi.[6,12] Next to methods that 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 of these synthetic-biology-based tools provide new alternatives to further aid the exploitation of fungal workhorses.

Targeted DNA delivery and precise genome editing are often required for the construction of STF-regulated genetic circuits. Engineering of nondomesticated strains is often time-consuming, and engineering efforts show low efficiency. The 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 (nondomesticated) fungal isolates, genome engineering can be less efficient because of the presence of the NHEJ machinery, resulting in more random integration events. In such strains, DNA delivery using nonintegrative fungal shuttle vectors can be advantageous, as this method does not rely on genomic integrations. The AMA1 sequence provides autonomous vector replication and therefore supports episomal DNA delivery in several species of filamentous fungi, and shuttle vectors 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 recombination of already available genetic parts or 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) are first inserted into entry vectors (level 0) to create genetic parts. These basic genetic parts (also called 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 a single plasmid, where the number of units is limited only by the

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Table 1. Genetic Modules and Other Vectors in the Fungal Toolkit for Modular Cloning (FTK)[a]

vector name	Addgene ID	part type	unit description	recipient MoClo backbone	template	source and references
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 ID 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
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
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

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Table 1. continued

vector name	Addgene ID	part type	unit description	recipient MoClo backbone	template	source and references
pFTK042	171314	CDS1	hisB AN6536.2 histidine, selection marker	pICH41308	pSE1.6	37
pFTK043	171315	CDS1	eGFP fluorescent reporter	pICH41308	pLM2_30 (Addgene ID 154222)	7
pFTK044	171316	CDS1	eGFP-NLS fluorescent reporter	pICH41308	pLM2_30 (Addgene ID 154222)	7
pFTK045	171317	CDS1	eGFP-SKL fluorescent reporter	pICH41308	pLM2_30 (Addgene ID 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_1147651 cP_mCherry	6
pFTK050	171322	CDS1	dTomato fluorescent reporter	pICH41308	pMF30.1	46
pFTK051	171323	CDS1	eBFP fluorescent reporter	pICH41308	pLM2_30 (Addgene ID 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 ID 46106)	40
pFTK055	171327	CDS1	rtTA2S-M2 (TetR-3xVP16) transcriptional activator	pICH41308	pVG2.2	5
pFTK056	171328	CDS1	SpCas9-NLS	pICH41308	pYTK036 (Addgene ID 65143)	14
pFTK057	171329	CDS1	dSpCas9(m4)-VPR-NLS	pICH41308	pYTK036 (Addgene ID 65143), pAG414GPD (Addgene ID 63801)	14, 47
pFTK058	171330	CDS2	QF DBD from QA-1F (for fusion)	pICH41258	pAC-7-QFBDAD (Addgene ID 46096)	40
pFTK059	171331	CDS2	LexA DBD (for fusion)	pICH41258	FRP718_PACT1(-1-520)-LexA-ER-haB42-TCYC1 (Addgene ID 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 ID 65143)	14, 21
pFTK062	171334	CDS2	dSpCas9(m2) (for fusion)	pICH41258	pYTK036 (Addgene ID 65143)	14, 21
pFTK063	171335	CDS2	dSpCas9(m4) (for fusion)	pICH41258	pYTK036 (Addgene ID 65143)	14, 21
pFTK064	171336	CDS3	QF AD from QA-1F (for fusion)	pAGM1299	pAC-7-QFBDAD (Addgene ID 46096)	7, 40
pFTK065	171337	CDS3	B42 AD (for fusion)	pAGM1299	FRP718_PACT1(-1-520)-LexA-ER-haB42-TCYC1 (Addgene ID 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 ID 47107)	47
pFTK069	171341	CDS4	eGFP-NLS fluorescent reporter (for fusion)	pAGM1301	pLM2_30 (Addgene ID 154222)	7
pFTK070	171342	CDS4	eYFP-NLS fluorescent reporter (for fusion)	pAGM1301	pLM2_30 (Addgene ID 154222) with S65G/V68L/S72A/T203Y mutations	7
pFTK071	171343	CDS5	eGPF-NLS fluorescent reporter (for fusion)	pICH41264	PX458 (Addgene ID 48138)	7
pFTK072	171344	CDS5	p300core HAT AD, *Homo sapiens* E1A binding protein p300 (for fusion)	pICH41264	pcDNA-dCas9-p300 (Addgene ID 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 ID 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

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Table 1. continued

vector name	Addgene ID	part type	unit description	recipient MoClo backbone	template	source and references
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] *A. niger* Pol-III terminator	pICH41276	*A. niger* N402	28
pFTK085	171357	T1	TtRNA[Met] *A. niger* Pol-III terminator	pICH41276	*A. niger* N402	28
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 (level 1)	pks17 Pc21g16000 (conidial pigment biosynthesis) *P. rubens* 5′ flanking region	pICH47732 (lvl1)	*P. rubens* DS54468	28
pFTK092	171364	TU (level 1)	pks17 Pc21g16000 (conidial pigment biosynthesis) *P. rubens* 3′ flanking region	pICH47772 (lvl1)	*P. rubens* DS54468	28
pFTK093	171365	TU (level 1)	sgRNA transcription unit (MoClo lvl1 unit), P-gpdA-HH-sgRNA-HDV-Ttrpc	pICH47761 (lvl1)	pFC334 (Addgene ID 87846), pLM-AMA18.0 dCas9-VPR (Addgene ID 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	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 ID 65143), pLM-AMA15.0 Cas9 (Addgene ID 138944)	14
pFTK096-LM-AMA018.0	171368	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 ID 65143), pAG414GPD (Addgene ID 63801), pLM-AMA18.0 dCas9-VPR (Addgene ID 138945)	14

[a]Units in the toolkit are described using a vector name, an 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).

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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 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 *Aspergillus 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 with regard to assembly compared with systems that leave an "assembly scar" after cloning. As the genetic parts in such systems are flanked with Type IIs restriction enzyme cut sites because 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 unit for cytosolic proteins consists of promoters (P), untranslated regions (U), coding sequences (CDS) and terminators (T), and four-base-pair linker sequences are used to connect them to each other and to the receiving backbone (*e.g.*, GGAG-(P)-TACT-(U)-AATG-(CDS)-GCTT-(T)-CGCT). This hierarchical structure provides a platform for rapid and easily automatable assembly of multigene constructs but on the other hand creates limitations for interchanging building blocks from other modular systems. Numerous modular assemblies have aimed to improve the standard MoClo assembly,[20,21,24] but by changing the linker sequences for transcription unit assembly and failing to consider backward compatibility, this creates incompatibility among 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 CDSs for transcriptional activation and DNA-binding domains, fluorescent reporters, and the AMA1 sequence for fungal autonomous replication as well as CRISPR components such as *Cas9*, *dCas9* sequences, and single guide RNA (sgRNA) 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.

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 among 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 of the vectors were constructed using the standardized MoClo system, which was discussed in detail by Weber *et al.*.[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.

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, three 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. 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 provide 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-phosphogluconate dehydrogenase) was shown to give an intermediate strength of transcription[27] and proven to be weaker than 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] Our toolkit also includes the constitutive promoter of *oliC31* (An04g08190, mitochondrial ATP synthase subunit 9), 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 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, and P*alcA* by aldehydes) are incorporated.

Synthetic Promoters. An increasing number of promoter libraries have been designed for yeast and filamentous fungi by the creation of synthetic promoters for STFs through the combination of various upstream activating sequence (UAS) elements and different core (or minimal) promoters (CPs).[6,7]

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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 UASs compatible with a DBD of an 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 similar to that of highest expressed native genes.[7] Moreover, entry vectors are provided for the construction of 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]

Synthetic Transcription Factors. Various 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). On the basis of 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 STFs, most commonly VP16 or its tandem repeats (VP64, VP160) from herpes simplex virus. Numerous DBDs of transcription factors have been 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] and the Gal4 and LexA DBDs, which are frequently used in synthetic expression systems. In *Aspergillus* species, 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 DBDs (from the qa-1F, Gal4, LexA, and TetR transcription factors) and transcriptional activation domains (from the qa-1F, Gal4, and B42 transcription factors), VP16 and its four tandem repeats VP64, the tripartite activator VPR (VP64-p65-Rta), and histone acetyltransferases (p300core and 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 *S. pyogenes*, which is the most widely applied Cas protein in filamentous fungi. Catalytically active Cas9 provides opportunities for genome editing, whereas dCas9 can be applied to deliver 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 the use of 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] The toolkit provides various options for CRISPR sgRNA delivery. 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, resulting in a transcript that 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 relies only on an established promoter in the host and ribozyme sequences that work across multiple species. Although the delivery of the ribosome-self-cleaved sgRNAs has been 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] Therefore, the toolkit provides entry vectors 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 insertion of the sgRNA target sequence into the sgRNA transcription unit, whereas 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/white 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, and VPR), histone acetyltransferases (p300core and Rtt109), and 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 a 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*, and *hisB*) as entry vectors. Table 1 shows DNA sources of the markers and their established applications. 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 *Aspergillus*, *Trichoderma*, and *Penicillium* species lacking acetamidase activity, overexpression of the acetamidase (*amdS*) gene provides selection on media containing acetamide as a sole nitrogen source that can be counterselected using fluoroace-

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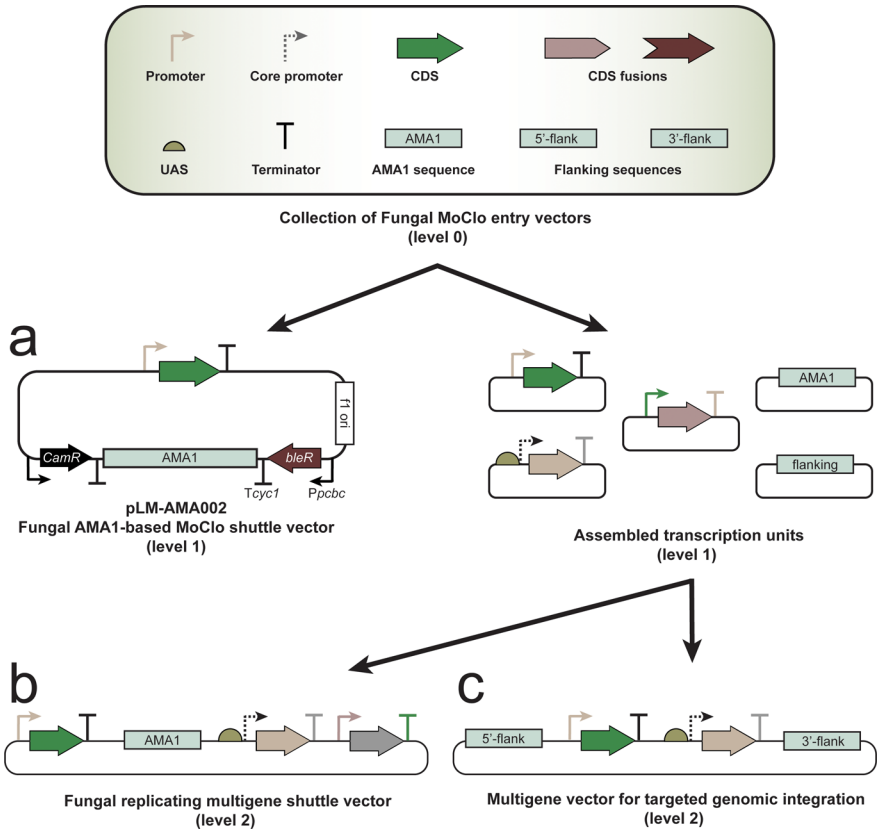


Figure 2. Transcription unit construction using the MoClo system and delivery platforms. A schematic representation of the recombination and assembly of the MoClo entry vectors into transcription units is shown. Transcription units can be assembled into (a) fungal shuttle vectors or (b, c) multigene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

tamide. The orotidine 5′-phosphate decarboxylase (*pyrG*) gene from *A. oryzae* is widely applied in *Aspergillus*, with examples in *Penicillium* and *Neurospora*, as a strong, recyclable, auxotrophic selection marker that can be counterselected using 5-fluoroorotic acid or fully supplemented using uracil or uridine. Overexpression of the bacterial resistance genes as phleomycin (*ble*) or 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 has been shown to play a role in conferring resistance to the fungicide carboxin in *A. flavus*. After generation of a histidine-auxotrophic strain, delivery of 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*.

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; without selection, more rapid loss of the vector was detected compared with 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 original bacterial vector into a fungal replicating episomal vector (Figure 2b). Fungal shuttle vectors can be assembled in *Escherichia coli* and delivered into *Aspergillus*, *Penicillium*, potentially 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 (Figures 2a and 3). As the assembly follows the MoClo language,[19] the vector uses BsaI restriction enzyme-generated GGAG and CGCT fusion sites to receive 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 convenient blue/white screening of successful clones. The 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

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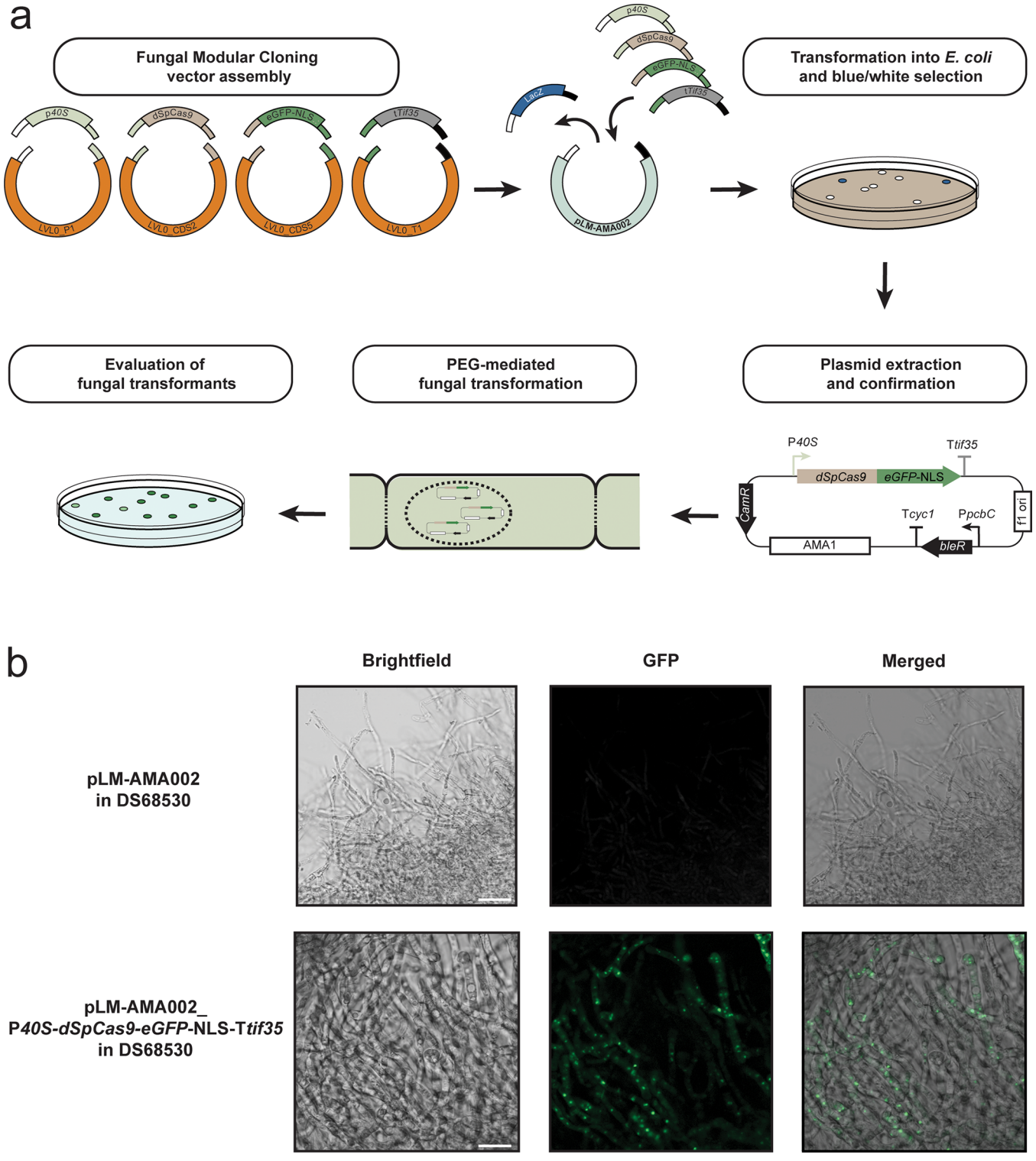


Figure 3. Transcription unit assembly from MoClo entry vectors on a pLM-AMA002 fungal shuttle vector and delivery to filamentous fungi. (a) Schematic representation of the assembly of MoClo entry vectors into a single transcription unit delivered to *P. rubens* on the pLM-AMA002 fungal shuttle vector.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

shuttle vector for expressing a gene of interest, a transcription unit was assembled that expresses a fusion protein of the catalytically dead Cas9 protein (dSpCas9) from *S. pyogenes* and a green fluorescent protein with SV40 nuclear localization (eGFP-NLS) 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 MoClo assembly (Figure 3a). The restriction-ligation-based assembly resulted in an AMA1 vector expressing a direct fusion of dSpCas9 and eGFP-NLS driven by a constitutive promoter. The created vector was delivered to *P. rubens*, and the expression of the protein fusion

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was validated using fluorescence microscopy, which showed expression of nucleus-localized GFP (Figure 3b). The construction of this expression platform required the integration of the coding sequence of the gene of interest into the appropriate position-predetermined MoClo entry vector. As numerous entry vectors from the toolkit can be utilized, 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 in later assembly steps. With the high efficiency of MoClo assembly, transcription units can be rapidly assembled in a single cloning step. Meanwhile, multigene genetic circuits can be constructed in two cloning steps (carrying up to seven transcription units per assembly).[19]

Taken together, this Fungal Modular Cloning Toolkit aims to accelerate 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 and inducible), terminators, activator- and DNA-binding-domains of transcription factors, fluorescent reporters, fungal selection markers, and CRISPR proteins (SpCas9 and dSpCas9) that are applicable for CRISPR-based applications. All of the vectors are built using the MoClo synthetic biology language, which allows the user to assemble numerous transcription units on a single plasmid that can later be delivered to the desired host organism by various delivery methods. To further accelerate the testing of functional transcription units, genetic parts are included that have been 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] and 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 assumes functionality in other filamentous fungal strains. The positions of the modular entry vectors in a transcription unit assembly are represented together with location identifiers in Figure 1. Complete vector sequences are available as Genebank files in Supplementary File S1 and available on Addgene as the "Fungal Toolkit for Modular Cloning (FTK)".

METHODS

Chemicals, Reagents, Oligodeoxyribonucleotides, and Cloning. All medium components and chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The 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 Diagnostics, Rotkreuz, Switzerland). Templates for PCR amplifications were acquired from various sources (Table 1) or ordered as synthetic DNA fragments from Thermo Fisher Scientific. 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 of the 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 that use 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 vector (P*40s* An0465 (P1), dSpCas9(m2) (CDS2), eGFP-NLS (CDS5), and T*tif35* (T1)) and the backbone vector pLM-AMA002.

The 50% shorter AMA1 sequence[25] was created by PCR and integrated into a MoClo entry vector. The autonomously replicating shuttle vector carrying the AMA1 sequence was based on the pDSM-JAK-109 backbone where the p*Gpda*-*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, to create 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 Table S1.

Fluorescence Microscopy. Transformants were further cultivated after transformation on phleomycin (50 μg/mL)-supplemented 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). Confocal imaging was performed on a Carl Zeiss LSM800 confocal microscope using a 20× 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 bandpass emission filter.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00260.

List of fungal strains used in this study and created strains with their corresponding transformed donor DNA and representation of different sgRNA transcription unit assembly methods (PDF)

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Elements of the Fungal Toolkit for Modular Cloning (FTK) as Genebank files (ZIP)

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

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

MoClo, Modular Cloning; BGC, biosynthetic gene cluster; NRPS, nonribosomal peptide synthetase; 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, activation domain

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