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MINI-REVIEW

Strategies for gene disruption and expression in filamentous fungi

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Abstract

Filamentous fungi can produce many valuable secondary metabolites; among these fungi, endophytic fungi play an ecological role in mutualistic symbiosis with plants, including promoting plant growth, disease resistance, and stress resistance. However, the biosynthesis of most secondary metabolites remains unclear, and knowledge of the interaction mechanisms between endophytes and plants is still limited, especially for some novel fungi, due to the lack of genetic manipulation tools for novel species. Herein, we review the newly discovered strategies of gene disruption, such as the CRISPR-Cas9 system, the site-specific recombination *Cre/loxP* system, and the I-*SceI* endonuclease-mediated system in filamentous fungi. Gene expression systems contain using integration of target genes into the genome, host-dependent expression cassette construction depending on the host, a host-independent, universal expression system independent of the host, and reporter-guided gene expression for filamentous fungi. Furthermore, the Newly CRISPRi, CRISPRa, and the selection markers were also discussed for gene disruption and gene expression were also discussed. These studies lay the foundation for the biosynthesis of secondary metabolites in these organisms and aid in understanding the ecological function of filamentous fungi.

Keywords Filamentous fungi · Gene disruption · CRISPR-Cas9 · Universal expression system · Reported-guided gene expression

Introduction

Fungi have the capacity to produce a vast number of valuable secondary metabolites, and some of them have an ecological function (Keller 2019), such as the PKS-NRPS hybrid metabolites revealed by genomics-driven discovery in *Aspergillus nidulans* (Bergmann et al. 2007); tenuazonic acid, which is produced by the rice blast fungus *Pyricularia oryzae* (Yun et al. 2017); and the polyketide equisetin, which is synthesized in *Fusarium heterosporum* (Kakule et al. 2015). In recent years, more fungal genomes have been sequenced with the further development of DNA sequencing. However, most of the gene clusters for secondary metabolites are silent or cryptic. Endophytes with a wide range of applications are becoming a hot spot popular area of research, and there is a growing interest in the exploration of endophytic fungi that can produce natural products (Vasundhara et al. 2016; Yan et al. 2018). Studies by Garyali indicated that five endophytes from *Himalayan yew* could produce tTaxol, a widely used natural anticancer drug (Garyali et al. 2014). Fifteen new polyketides were revealed in the endophytic fungus *Pestalotiopsis fici* (Zhou et al. 2019). On the other hand, some endophytic fungi could promote plant growth, enhance disease resistance, elicit a defense response, and act as remediators of abiotic stress (Khare et al. 2018; Jia et al. 2016). For example, the two endophytic fungal *Phoma glomerata* LWL2 and *Penicillium* sp. LWL3 could secrete indoleacetic acid (IAA) and increase the biomass of host plants (Waqas et al. 2012). *Phomopsis liquidambari* is an endophytic fungus with the ability to enhance nitrogen uptake and metabolism and promote rice growth and yield when inoculated into rice (Yang et al. 2014). It could also induce nodulation in peanut and increase nitrogen fixation by activating auxin signalling (Zhang et al. 2017b; Zhang et al. 2018b). The volatile organic compounds produced by *Hypoxylon anthochroum* showed antifungal activity and were potential mycofumigation agents against postharvest diseases caused by *Fusarium oxysporum* (Macías-Rubalcava et al. 2018). *Trichoderma viride* and *Trichoderma harzianum* dissolve the soil and can induce a range of plants to produce local and systemic resistance to a variety of plant pathogens, triggering the plant's defense response and

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increasing nutrient absorption and nitrogen use efficiency (Harman et al. 2004). These studies suggest that filamentous fungi play an important role in the discovery of novel secondary metabolites and in ecological applications. However, most studies of the new secondary metabolites of fungi and interaction mechanisms between fungi and plants are still challenging because genetic manipulation and molecular biological tools are usually difficult to apply in filamentous fungi. The main limiting factors are as follows: (I.) Filamentous fungi have a more complex genetic background than those of yeast and bacteria. (II.) The efficiency of homologous recombination in filamentous fungi is very low, usually less than 5% (Kück and Hoff 2010). (III.) The available screening markers are limited, and not all screening markers are effective for filamentous fungi.

Gene expression is also important for filamentous fungi involved in biotechnological processes, such as *Trichoderma reesei*, *Aspergillus niger*, and *Aspergillus oryzae* (Druzhinina and Kubicek 2017; Steiger et al. 2018; Guerrero et al. 2019). Novel hosts are usually those with superior native characteristics, such as high resistance to extreme conditions (Khan et al. 2015), specific metabolic traits (Rahnama et al. 2018), and efficient protein secretion (Zhang et al. 2017c), which could offer interesting opportunities for different microbes. However, the lack of suitable expression systems has slowed the development of each individual organism and hindered the use of novel hosts. Plasmids and host strains for gene expression in filamentous fungi are less commercially available for filamentous fungi than for yeast and *Escherichia coli*, so such expression systems must be constructed in the laboratory. To date, a few gene expression strategies have been reported, providing some prospects for filamentous fungi.

Although molecular tools for gene manipulation have been reported in filamentous fungi (Wang et al. 2017a), new techniques are ceaselessly developed. Thus, we reviewed the newly developed gene disruption and gene expression tools in reported filamentous fungi. This will be helpful for future the research on silent gene clusters related to secondary metabolites and the study of interaction mechanisms between filamentous fungi and hosts in the future.

Construction of a gene knockout system in filamentous fungi

CRISPR-Cas9 system

CRISPR-Cas9 (clustered regularly interspaced short palindromic repeat-associated RNA-guided DNA endonuclease Cas9) systems originated from bacterial adaptive immune systems. The system has rapidly become a widely used technology due to its high efficiency and straightforward design and has been used in organisms (Yao et al. 2018; Grzybek et al. 2018; Bao et al. 2019). The Cas9 endonuclease is guided by a gRNA to a specific locus, where it introduces a double-strand break (DSB) in the DNA sequence. The protospacer of the gRNA that defines the target DNA can be virtually any 17–20-bp nucleotide sequence found adjacent to a 5′-NGG DNA motif (the PAM, protospacer-adjacent motif). There are two cell repair mechanisms to fix the DSB introduced by the Cas9 nuclease. One is the error-prone nonhomologous end-joining (NHEJ) mechanism, which sometimes leads to insertions or deletions within the target sequence that typically cause a dysfunctional open reading frame (ORF). The other is homologous recombination (HR) with homology close to the DSB (a so-called donor DNA, dDNA) (Sander and Joung 2014). Thus, the CRISPR/Cas9 system can be used for the creation of gene deletions and insertions in filamentous fungi, as shown in Table 1. CRISPR-Cas9 systems have made great progress in filamentous fungi, and mostly of them are in model fungi (Shi et al. 2017; Sander and Joung 2014). Although more endophytic fungi have been found to play important roles in the discovery of secondary metabolites, ecological functions, or agricultural applications (Chen et al. 2016; Liao et al. 2019), there are fewer CRISPR-Cas9 systems for them. Herein, we review the following three main strategies for the expression of Cas9 and gRNA: Cas9 in vivo and gRNA both in vivo, Cas9 in vivo and gRNA in vitro, and Cas9 in vitro and gRNA both in vitro (Fig. 1a). These strategies will provide a path for CRISPR-Cas9 systems for nontraditional fungi. Then, a vector or RNP complex was transformed into filamentous fungi by protoplast-mediated transformation (PMT) or Agrobacterium-mediated transformation (AMT) (Fig. 1b). Finally, the disruption of the target gene would be finished completed using NHEJ or HR via both gRNA- and Cas9-mediated DSB (Fig. 1c).

Cas9 in vivo, gRNA in vivo

Both Cas9 and gRNA in vivo have been widely used in many filamentous fungi in vivo. They require the construction of expression cassettes for the expression Cas9 and gRNA. Cas9 was either integrated into the genome or expressed from a plasmid that was presumably not integrated into the genome in filamentous fungi (Liu et al. 2015; Arazoe et al. 2015; Matsu-Ura et al. 2015; Schuster et al. 2016; Fuller et al. 2015; Nødvig et al. 2015; Katayama et al. 2016; Zhang et al. 2016; Song et al. 2018b; Nielsen et al. 2017), which was shown to increase gene knockout and HDR (homologous directed recombination) events up to high efficiencies when gene deletions were obtained through the introduction of homologous arm dDNAs. gRNA expression was usually key for this strategy because RNA polymerase III promoters could not be recognized by filamentous fungi and were not suitable for all microorganisms. RNA polymerase III promoters such as SNR52 and some tRNA promoters can be used to transcribe in

Table 1 Application of CRISPR-Cas9 system in filamentous fungi

Groups	Species	Cassette	Selection marker	Principle	Transformation	Efficiency	Refs
Cas9 in vivo, gRNA in vivo	*Pyricularia oryzae*	U6p-sgRNA-T5t-TEFp-Cas9-GLAt	Bar	HR	PMT	High	Arazoe et al. 2015
	Neurospora crassa	TrpCp-Cas9-SV40NLS-trpCt,SNR52p-gRNA-SUP4t	Bar/leu/ura	HR	Electroporation	High	Matsu-Ura et al. 2015
	Ustilago maydis	U6p-sgRNA-U6t-TEFp-NLS-Cas9-HA-NLS-NOSt	Carboxin	NHEJ	PMT	70~100%	Schuster et al. 2016
	Aspergillus fumigatus	TtrpC-hph-PgpdA-Ptef1-Cas9-SV40-NLS-Psnr52-gRNA-Tsu	hyg/ble	NHEJ	PMT	25 to 53%	Fuller et al. 2015
	Aspergillus niger	AMA1-PgpdA-HH ribozyme-sgRNA-HDV ribozyme-TtrpC-Ptef1-Cas9-Ttef1	pyrG/ble/argB/hyg	NHEJ/HR	PMT	70–80%	Nødvig et al. 2015
	Aspergillus oryzae	amyBp-FLAG-NLS-Cas9-NLS-amyBt-U6p-sgRNA-U6t-niaD	nia	NHEJ	PMT	10–20%	Katayama et al. 2016
	Aspergillus niger	AMA1-tRNAp-gRNA-tRNAt-pkiAp-Cas9-NLS-glaAt	pyrG/ble	HR	PMT	~ 90%	Song et al. 2018
	Talaromyces atroroseus	AMA1-PgpdA-HH ribozyme-sgRNA-HDV ribozyme-TtrpC-Ptef1-Cas9-Ttef1	hyg	HR	PMT	Not given	Nielsen et al. 2017
	Aspergillus carbonarius	pGpdA-HH-Tra:crRNA-HDV-Trpc-AMA1-pTef-Cas9-NLS-tTef	hyg	HR	PMT and AMT	3–27%	Weyda et al. 2017
	Sclerotinia sclerotiorum	U6p-sgRNA-T5t-TEFp-Cas9-GLAt	hyg	NHEJ	PMT	41%	Li et al. 2018
	Myceliophthora thermophila	Not given	hyg/bar/neo	HR	PMT	Not given	Gu et al. 2018
	Rhizopus delemar	partial:tRNAP-gRNA scaffold-TEFP-3×FLAG-NLS-Cas9	pyrF	NHEJ	Electroporation	Not given	Bruni et al. 2018
	Fusarium graminearum	Ptef-HH ribozyme-gRNA-HDV ribozyme,PgpdA-Cas9-TTRI4	hyg	MMEJ/NHEJ	PMT	Not given	Gardiner and Kazan 2018
	Alternaria alternata	AMA1-PgpdA-HH ribozyme-sgRNA-HDV ribozyme-TtrpC-Ptef1-Cas9-Ttef1	hyg/pyrG	NHEJ	PMT	25%	Wenderoth et al. 2017
	Aspergillus oryzae	amyBp-FLAG-NLS-Cas9-NLS-amyBt-U6p-sgRNA-U6t-niaD	niaD/pyrG	NHEJ	PMT	60~80%	Nakamura et al. 2017
	Aspergillus niger	PglaA-NLS-Cas9-NLS-TglaA,PhU6/PyU6/PanU6-sgRNA-T6	amds	NHEJ/HR	PMT	15–23%	Zheng et al. 2018a
	Aspergillus niger	PglaA-NLS-Cas9-NLS-TglaA,5SrRNA/PhU6-HDV-sgRNA-T6	amds	HR	PMT	100%	Zheng et al. 2018b
	Aspergillus carbonarius	pGpdA-HH-Tra:crRNA-HDV-Trpc-AMA1-pTef-Cas9-NLS-tTef	hyg	HR	PMT and AMT	3–27%	Weyda et al. 2017
	Myceliophthora thermophila	U6p-sgRNA-tttttttt,Tef1p-3×FLAG-NLS-Cas9-NLS-TrpCt	neo	NHEJ	PMT	Not given	Xu et al. 2018
	Aspergillus oryzae	PU6-sgRNA-TU6-halfAMA1-PamyB-FLAG-NLS-Cas9-NLS-TamyB	pyrG	HR	PMT	55.6–100%	Katayama et al. 2018
	Aspergillus fumigatus	PgpdA-HH-ribozyme-sgRNA-HDV-ribozyme-term; TtrpC-Ptet^{on}-Cas9-Ttef	hyg/pyrG/tynC	HR	PMT	80%	Weber et al. 2017
	Aspergillus niger	AMA1-based pCas	hyg/pyrG	NHEJ	PMT	Highest100%	Sarkari et al. 2017
	Myceliophthora thermophila	U6p-sgRNA-tttttttt,Tef1p-3×FLAG-NLS-Cas9-NLS-TrpCt	bar/amds	NHEJ/HR	PMT and AMT	22%	Liu et al. 2017
	Aspergilli nidulans/Aspergilli niger/Aspergilli oryzae	PAf_U3-tRNA-sgRNA-tRNA-TAf_U3,Cas9-AMA1-Marker	argB/pyrG/hyg/ble	HR	PMT	10–100%	Nødvig et al. 2018
	Penicillium chrysogenum	Xyl-hCas9-Xyl-RNAIII-sgRNA-RNAIII-gpdA-amdS-at-AMA1	acetamide	HR	PMT	Not given	Pohl et al. 2016
Cas9 in vivo, gRNA in vitro	*Aspergillus niger*	AMA1-PgpdA-HH ribozyme-sgRNA-HDV ribozyme-TtrpC-Ptef1-Cas9-Ttef1	hyg/ura	HR	PMT	27.5–100%	Kuivanen et al. 2016
	Trichoderma reesei	In vitro RNA transcription, Ppdc- Cas9-eGFP-Tpdc	hyg/cefotaxime	HR	PMT and AMT	93–100%	Liu et al. 2015

Table 1 (continued)

Groups	Species	Cassette	Selection marker	Principle	Transformation	Efficiency	Refs
	Aspergillus fumigatus	In vitro RNA transcription, AMA1-PgpdA-3× FLAG-NLS-Cas9-NLS-Ttrpc	hyg/pyrG	HR	PMT	~ 95–100%	Zhang et al. 2016
	Nodulisporium sp.	pBSKII-PtrPC-neo-TtrPC-U6 Nod-gRNA-g3279, pBSKII-PtrPC-neo-TtrPC-U6 Asp-gRNA-g3279, pBSKII-PtrPC-Flag-toCas9-TtrPC	hyg	NHEJ	PMT	68.30%	Zheng et al. 2017
	Penicillium chrysogenum	In vitro RNA transcription and Xyl-hCas9-Xyl-gpdA-amdS-at-AMA1	acetamide	HR	PMT	23%	Pohl et al. 2016
Cas9 in vitro, gRNA in vitro	*Penicillium chrysogenum*	In vitro RNPs	acetamide	HR	PMT	3.9%	Pohl et al. 2016
	Aspergillus fumigatus	In vitro RNPs	hyg	HR	PMT	Not given	Al-Abdallah et al. 2018
	Fusarium oxysporum	In vitro RNPs	ura	NHEJ	PMT	21.40%	Wang et al. 2018
	Aspergillus fumigatus	In vitro RNPs	hyg	HR	PMT	97–100%	Al-Abdallah et al. 2017
	Mucor circinelloides	In vitro RNPs	Color	NHEJ/HR	PMT	0% or 100%	Nagy et al. 2017
	Trichoderma reesei	In vitro RNPs	pyr4	HR	PMT	30%	Hao and Su 2019
	Magnaporthe oryzae	In vitro RNPs	hyg	NHEJ /HR	PMT	> 70%	Foster et al. 2018

PMT, protoplast-mediated transformation; *AMT*, *Agrobacterium*-mediated transformation

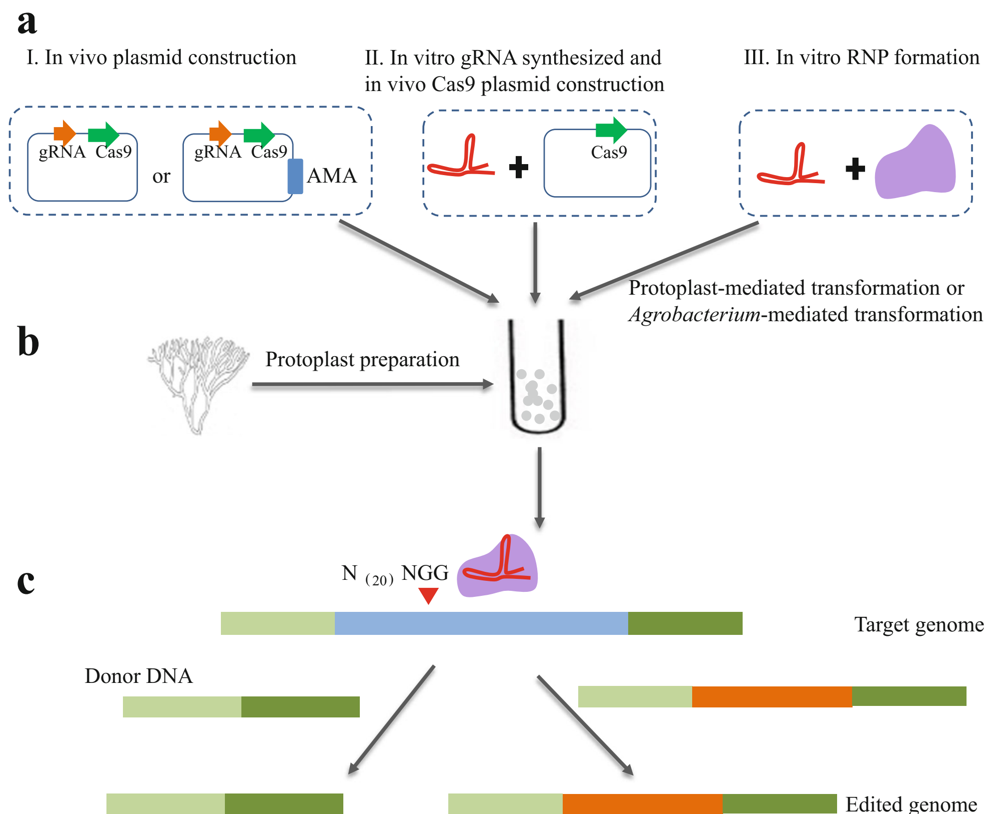

Fig. 1 The principle of CRISPR-Cas9-mediated gene disruption. a I, Expression vector of the Cas9 and gRNA cassette in vivo without an AMA element and expression vector of the Cas9 and gRNA cassette in vivo containing an AMA element; II, construction of the expression vector of Cas9 in vivo and the synthesized gRNA in vitro; III, formation of the RNP complex of Cas9 and gRNA in vitro. b The vector or RNP complex was transformed into the protoplast of filamentous fungi by PMT or AMT. c The disruption of the target gene occurs via both gRNA- and Cas9-mediated DSB and repair using NHEJ or HR

fungi such as *Neurospora coarsens*, *Aspergillus fumigatus*, and *Aspergillus niger*, and the efficiency of gene mutation was as high as 97% (Matsu-Ura et al. 2015; Fuller et al. 2015; Song et al. 2018a). In addition, the endogenous or heterologous *U6* promoter of small nuclear RNA (snRNA) has often been used to express gRNAs in various organisms (Nielsen et al. 2017; Nakamura et al. 2017; Zheng et al. 2018a; Zheng et al. 2018b; Xu et al. 2018; Katayama et al. 2019), some of which experienced highly efficient gene mutation at the targeted loci using donor DNAs with homologous arms as short as 40 bp (Zheng et al. 2018a). The use of the 5S rRNA gene as a gRNA promoter could be broadly applied for engineering eukaryotic CRISPR/Cas9 toolkits (Zheng et al. 2018b). However, some promoters of endogenous RNA polymerase III from filamentous fungi are currently difficult to identify or unsuitable for gRNA transcription. Therefore, the most common way to express gRNA in vivo was to use two ribozyme sequences, those of 5′ terminal hammerhead (HH) and 3′ terminal hepatitis delta virus (HDV), located on the flank of the gRNA (Wenderoth et al. 2017; Weyda et al. 2017). Interestingly, the gRNAs for *P. oryzae*, their gRNAs could be successfully transcribed using the RNA polymerase II promoters 35S CAMV promoter and Trpc promoter without two ribozyme sequences in filamentous fungi (Arazoe et al. 2015). Although the strategies do not need to consider the stability and uptake of gRNA, Cas9 is active after translation and is relatively highly expressed, which may lead to off-target effects and toxicity to cells. To reduce these problems, the gene expression of Cas9 could be controlled by an inducible Tet-ON promoter by and the addition of doxycycline to *Aspergillus fumigatus* akuBKU80 (Weber et al. 2017). In most systems, a resistant or auxotroph selection marker is necessary. Most genetic tools are about for model strains, especially endophytic fungi. A split-marker gene deletion system for the model grass endophytic fungus *Epichloë festucae* or and insertional mutagenesis of the endophytic fungi *Kabatiella zeae* and *Calcarisporium arbuscula* was also developed by using HR and *Agrobacterium*-mediated transformation (AMT) (Rahnama et al. 2017; Sun et al. 2018; Cao et al. 2018). The resistant and auxotroph selection system may be suitable for the study of secondary metabolites, but not suitable for the studying the interaction between endophytic fungi and their hosts. Marker-free gene editing is most likely applicable for endophytes, which maybe reduce their influence on plants.

Some research has reported on marker-free gene editing and multiple gene editing in filamentous fungi. A CRISPR/Cas9 system including an AMA1-based autonomously replicating plasmid allowed for efficient gene deletion/integration and was used in filamentous fungi (Song et al. 2018b; Nielsen et al. 2017; Sarkari et al. 2017) and the endophyte *Alternaria alternata* (Wenderoth et al. 2017). Cas9 was transiently expressed with an inducible promoter or by transient plasmid propagation, and plasmids containing AMA1 were easily lost without drug resistance stress and an inducible promoter,

which led to rapid plasmid loss after transformation. Therefore, the CRISPR/Cas9 system could be easily removed for traceless screening after gene editing by the Cas9 protein and gRNA was complete. Forced recycling of the gene-editing plasmid for highly efficient marker-free multiple gene deletion/integration was developed in *A. oryzae* and *Penicillium chrysogenum,* which enables unlimited repeatable genetic engineering and facilitates multiple gene modification of fungal strains without the construction of auxotrophic strains prior to genome editing (Katayama et al. 2019; Pohl et al. 2016). Unstable AMA1 vectors for selection or genetic elements could be removed or integrated using dDNAs without genomic integration of a selection marker. This strategy is suitable for studies of the interaction between endophytes and host plants. Multiplexing and automated genetic engineering are still a bottleneck for studies of most filamentous fungi. The main limitations are evaluation of gRNA efficiency and generation of multiple different gRNA species simultaneously in filamentous fungi, although a tool to assess protospacer efficiency was established by exploiting a polymerase III promoter and tRNA sequences as spacers for the release of multiple different gRNAs for an efficient marker-free method (Nødvig et al. 2018). We suggest that genetic manipulation tools should be two systems for endophytes. One is for the study of secondary metabolites, which can draw on experiences from traditional model strains, and the resistance and auxotrophic selection system may be suitable for the study of secondary metabolites. The other system is for the interaction studies with endophytes and plants, which is proper using marker-free gene editing. It should be considered that the resistance and auxotrophic selection system may influence the growth and physiology of plants, and it is not easy to control when endophytic fungi colonize plants.

Cas9 in vivo, gRNA in vitro

gRNA was expressed under a polymerase II promoter, and additional ribozyme structures were required to release a functional gRNA due to the poor availability of characterized RNA polymerase III promoters. The system using Cas9 in vivo and gRNA in vitro is another strategy for gene disruption. The gRNA synthesized in vitro was cotransformed with a Cas9 vector into protoplasts of *A. nige*r, and then, the catabolism of galactaric acid was disrupted; an efficient galactaric acid-producing strain was generated via short deletions by NHEJ-mediated repair (Kuivanen et al. 2016). This approach overcame the time-consuming steps for using gRNA expression cassettes and other possible problems in gRNA expression and has also been used in *T. reesei* (Liu et al. 2015), *A. fumigatus* (Zhang et al. 2016) and *P. chrysogenum* (Pohl et al. 2016). Most Cas9 genes were highly transcribed by constitutive promoters to improve gene editing efficiency. Some inducible promoters (for example, Pcbh1 and PniiA) have been used to inhibit the overexpression of Cas9 to reduce the mistargeting effect or toxicity to cells, which further enhanced the controllability of the CRISPR/Cas9 system in filamentous fungi (Liu et al. 2015; Pohl et al. 2016). An efficient CRISPR-Cas9-based gene disruption strategy by simultaneous transformation of in vitro transcriptional gRNA and a linear marker gene cassette into Cas9-expressing fungi was developed. The linear marker gene cassette allows for the selection of transformants and enhances the gene disruption efficiency in *A. oryzae* NSAR1 and *Sporormiella minima* (Zheng et al. 2017). Combining the delivery of in vitro-synthesized gRNA with plasmid-based Cas9 expression may be the most versatile and rapid option for novel targets, as gRNA templates can be ordered as oligonucleotides and the simultaneous use of multiple gRNAs is easy. No additional cloning was needed when Cas9 was synthesized in vivo and gRNA was synthesized in vitro. The increased expression levels of Cas9 may lead to off-target effects and toxicity to cells. Therefore, controllability of the CRISPR/Cas9 system is necessary for filamentous fungi. In addition, it must overcome the stability of gRNA and maintain gRNA uptake.

Cas9 in vitro, gRNA in vitro

In recent years, researchers have developed a method for assembling the Cas9-gRNA complex in vitro because the intracellularly expressed Cas9 usually causes unexpected off-target gene disruption or toxicity to cells. In vitro, Cas9 and gRNA were transcribed together and subsequently formed an editor of the Cas9-gRNA ribonucleoprotein complex-targeting gene in *P. chrysogenum* (Pohl et al. 2016). When cotransformed with a DNA template, the CRISPR/Cas9 system resulted in ~ 42% efficiency of gene replacement with NHEJ machinery (kusA+). A CRISPR/Cas9 system that employed in vitro assembly of Cas9 ribonucleoproteins (RNPs) coupled with microhomology repair templates for gene deletion was effective in *A. fumigatus* (Al Abdallah et al. 2017). This system could also be applied with high-fidelity or rationally engineered Cas9 enzymes with increased specificity or by application of truncated versions of gRNAs according to previous reports (Kleinstiver et al. 2016; Slaymaker et al. 2016; Fu et al. 2014). The Cas9 ribonucleoprotein for Cas9 protein and gRNA was assembled to form a stable RNP in vitro, and this complex was then transferred into fungal protoplasts of pathogenic *F. oxysporum* using protoplast-mediated transformation (PMT) (Wang et al. 2018b). In vitro-assembled Cas9 RNPs coupled with microhomology repair templates were shown to be an efficient and universal system for gene manipulation in *A. fumigatus* (Al et al. 2017). The Cas9 enzyme and the gRNA complex were transferred into the Mucoral fungus *Mucor circinelloides* without in vitro RNP formation and the use of plasmids to disrupt the *carB* and the *hmgR2* genes. This method only required the design of a protospacer sequence and synthesis of the crRNA and

tracrRNA (Nagy et al. 2017). The in vitro-assembled ribonucleoprotein complex of Cas9 and gRNA was transformed with a plasmid containing the *pyr4* marker gene into *T. reesei* QM9414, which successfully disrupted the *cbh1* gene (Hao and Su 2019). These results suggested that direct transformation of the RNP complex into the cell is a rapid method to disrupt a gene, which may have wide applications in functional genomics research and can potentially reduce the toxicity to cells (Foster et al. 2018). Using an RNP complex for endophytes keep blank. The RNPs based on genome editing had the benefit of transient exposure of the cells to Cas9, which was usually highly toxic to cells; then, the RNPs would be degraded, reducing the chance of off-target events. No additional cloning was needed when both Cas9 and gRNA were synthesized in vitro. The expression levels of Cas9 were low and transient. The gRNA may be protected from degradation after forming a complex with Cas9, which leads to fewer mistargets. Moreover, transformation of Cas9 RNPs may be applicable in fungi that lack previously established molecular biology tools.

CRISPR-Cas9 has achieved great progress in many fungi. However, homozygous deletion is still a challenge for some filamentous fungi that are diploid or polyploid. Recently, a new gene drive system was engineered to obtain homozygous deletions using the CRISPR-Cas9 system in *Candida albicans* (Shapiro et al. 2018). Although it is associated with yeast, this system provides a new perspective for filamentous fungi. First, a plasmid containing Cas9 and donor DNA consisting of two small gRNAs flanked by homologous arms upstream and downstream of the ORF targeted for deletion was constructed (Fig. 2a). Then, Cas9 was targeted by the gRNAs to regions 5′ and 3′ of the ORF-induced DSBs when the plasmid was transformed into *C. albicans*. The DSBs were repaired through HR using the flanking sequences of the gRNA on the plasmid as a selfish genetic element, resulting in a mutant (*orf∆*) (Fig. 2b). The mutant orf∆ was mated to a wild-type strain containing the copy of the same ORF. Then, the cells propagated to replace additional wild-type loci, producing a homozygous double-gene deletion mutant of the ORF (*orf∆/orf∆*) because the gene drives further propagation into the incoming wild-type locus during mating (Fig. 2c). This strategy provides the rapid generation of homozygous deletion mutants in the diploid organism. This finding sheds new light on the homozygous deletion of filamentous fungi.

Site-specific recombination *Cre/loxP and FLP/FRT* system

Gene manipulation is a difficult task due to the complicated genetic background and limited availability of reliable selection markers in filamentous fungi. The *Cre/loxP* system derived from bacteriophage P1 is another powerful gene editing tool that has been used in gene function research and removal of available selection markers in eukaryotic organisms (McLellan et al. 2017). The *Cre/loxP* system has been used in filamentous fungi to overcome the limitations of available selectable marker genes, and the corresponding principle is shown in Fig. 3. In this system, a cassette containing the left homologous arm, loxP sites, resistance (R) gene, and right homologous arm was constructed with replication (Fig. 3a), and a fragment with the selectable marker R gene and *lox*P was integrated into the target genome (Fig. 3b). Then, the expressed *Cre* recombinase can specifically recognize a 34-bp *loxP* sequence and catalyze reciprocal recombinations between pairs of them, so that the whole digested region, including the target gene flanked by loxP, was cut off, and the original genome was recombined (Fig. 3d). The selection marker can be recycled in subsequent rounds of transformation due to the removal of a selectable marker gene in *Ashbya gossypii*, *Neotyphodium coenophialum*, *Neotyphodium uncinatum*, and *E. festucae* (Aguiar et al. 2014; Florea et al. 2009; Zhang et al. 2013). A marker recycling system for *T. reesei* was also developed based on the *Cre/loxP* system that allowed for highly efficient gene targeting using a strain with a deleted *tmus53* gene, which encodes a DNA repair and recombination protein (Steiger et al. 2011). Deletion strain, a strain with a deleted *ligD* gene, which encodes an NHEJ DNA repair protein, was constructed to optimize homologous recombination with the *Cre-loxP* system in *A. oryzae* (Mizutani et al. 2012; Zhang et al. 2017a). A genetic manipulation system was constructed by combining the *Cre*-lox*P* system with the Tet-on system in *Penicillium oxalicum* JUA10-1 (Jiang et al. 2016). The two genes, *ligD* and *bglI*, were sequentially disrupted by *loxP*-flanked *ptrA* using *Cre* recombinase driven by doxycycline and doxycycline controlled by the Tet-on system. A *Cre/loxP*-based system for *A. niger* as a platform strain was developed and has potential as a cell factory to produce variant organic acids (Park et al. 2017; Forment et al. 2006). Cre expression is driven by xylose and *loxP*-hph-*loxP* recombination driven by doxycycline. An efficient malate-producing strain was constructed by three-step genetic manipulation (deletion of *oah A*, insertion of *pyc* and *mdh*) in *A. niger* WU-2223 L. A series of vectors was created to allow for antibiotic selection (G418, nat1 or hphB) of transformants and subsequent negative selection for marker removal using thymidine kinase fusions combined with the *Cre/loxP* system in *Fusarium graminearum* (Twaruschek et al. 2018); the selection marker was subsequently removed using *Cre* recombinase and 2′-deoxy-5-fluorouridine. A *Cre/loxP*-mediated δ-integration system in *Saccharomyces cerevisiae* was versatile for sequential and simultaneous integration of two or more genes (Choi and Kim 2018).

Similarly, a *FLP/FRT* system derived from the yeast *S. cerevisiae* consisting of *FLP* recombinase and corresponding *FRT* recognition sites was first used in the filamentous fungi *P. chrysogenum* and *Sordaria macrospora* (Kopke et al. 2010). Every *FRT* had two 13-bp *FLP*-binding sites and was interrupted by an 8-bp spacer region, so the system needed two *FLP* recombinases to bind to one *FRT* sequence. If DNA strand breakage occurred, the overhanging ends of the

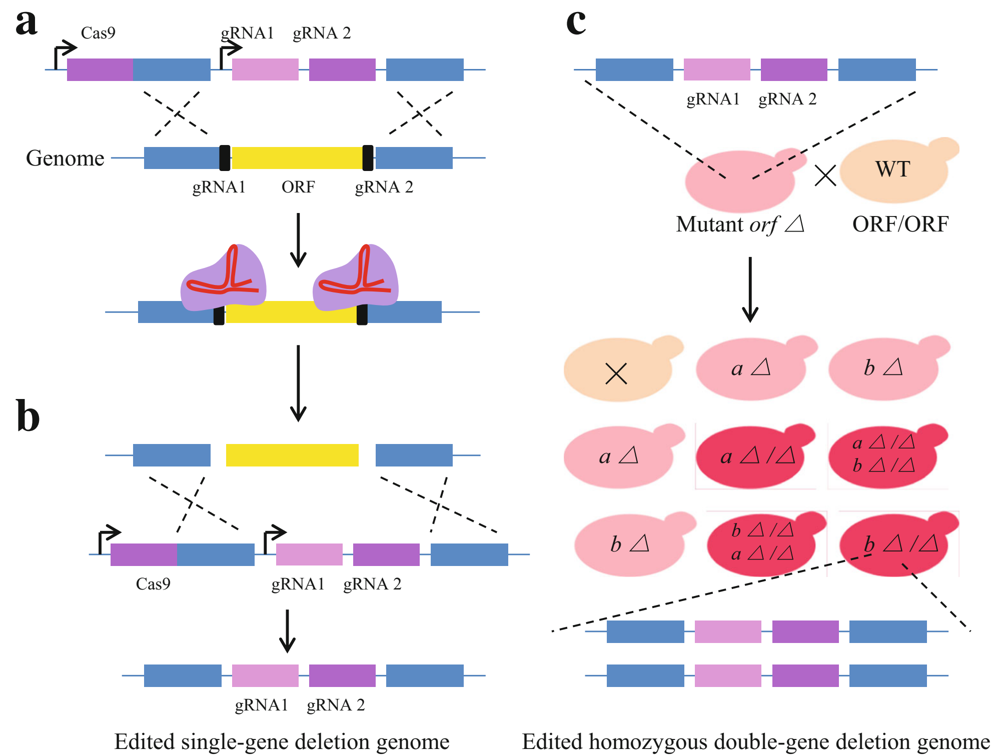

Fig. 2 Cas9-mediated system for targeted homozygous deletion in *C. albicans*. a Cas9 and donor DNA consisted of two small gRNAs flanked by homologous arms upstream and downstream of the ORF targeted for deletion. b Cas9 targeted by the gRNAs to regions at the 5′ and 3′ of the ORF-induced DSBs and then the DSBs were repaired through HR, using the flanking sequences of the gRNA on the plasmid as a selfish genetic element, resulting in mutant (*orf*Δ). c The mutant *orf*Δ was mated to wild-type strain containing the copy of the same ORF, then the cells propagated to replace additional wild-type loci, producing homozygous double-gene deletion mutant of the ORF (*orf*Δ/*orf*Δ) during mating

two FRT fragments produced 8-bp overhanging ends, and then a recombinant *FRT* sequence was generated by complementary base pairing. This method was used to allow gene deletion using a selectable marker (HygR) in *Ustilago maydis* (Khrunyk et al. 2010) and *Acremonium chrysogenum* (Bloemendal et al. 2014). The *FLP* recombinases were under the control of an arabinose-inducible promoter and a xylose-inducible promoter, respectively. The two systems proceeded

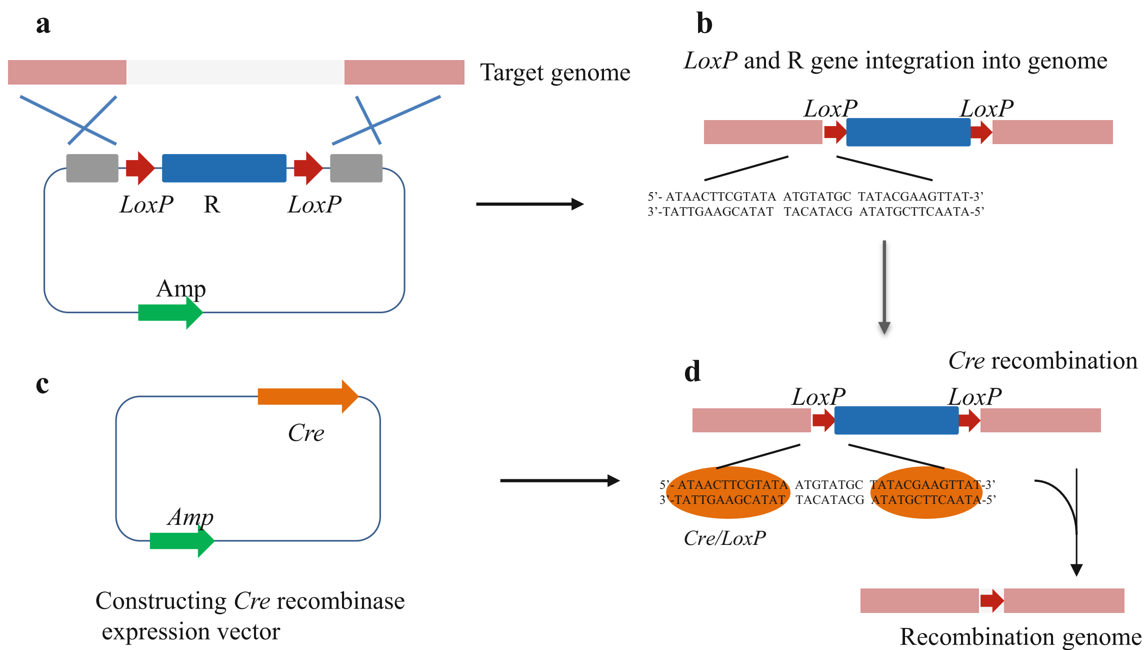

Fig. 3 The principle of site-specific recombination *Cre/loxP*. a The cassette containing left homologous arm, *loxP* sites, and resistance (R) gene and right homologus arm was connected into vector with replication. b The fragment with R gene and *LoxP* was integrated into target genome. c The *Cre* recombinase was expressed which would anchor the *LoxP* sites of the genome. d *Cre* recombinase digested the *LoxP* sites, so that the whole region digested including the target gene flanked by loxP was cut off and the original genome is recombined

through two steps to achieve gene deletion. First, a marker gene needs to be integrated into the host genome, and then the *Cre* or *FLP* is expressed to finish the gene deletion. Thus, the *Cre/loxP* system and the *FLP/FRT* system have been adapted for use in filamentous fungi and are effective tools for recycling marker genes and overcoming the limitations of available selection marker genes.

I-*Sce*I endonuclease-mediated system

The yeast endonuclease I-*SceI*, as a recipient-specific inducer to mediate DSBs, was used in *T. reesei* (Ouedraogo et al. 2015; Ouedraogo et al. 2016), *P. oryzae* (Arazoe et al. 2014), and *A. oryzae* (Takahashi et al. 2012). A schematic representation of the targeted integration of the gene of interest (GOI) expression cassette via I-*Sce*I enzyme-mediated homologous recombination is shown in Fig. 4. A reporter strain of *T. reesei*, which is uridine auxotrophic and had a nonfunctional GFP, was engineered by inserting two I-*Sce*I restriction sites at the *cbh2* locus, which produced a uridine prototrophic strain containing two I-*Sce*I sites surrounding the *pyrG* cassette or a reporter strain of *P. oryzae* (Fig. 4a). The expression of I-*Sce*I generated two DSBs and caused the loss of the *pyrG* selection marker between the I-*Sce*I sites after both the GOI-expressing cassette and the I-*Sce*I enzyme were cotransformed into the reporter strain to create DSBs (Fig. 4b). The DSB could be repaired by homologous recombination with the GOI-expressing cassette or GFP, which had regions homologous to the locus containing the I-*Sce*I sites (Fig. 4c). The mutant strain (lost *pyrG* gene) with a functional GFP was reconstituted after homologous recombination. In *P. oryzae*, this system consisted of donor and recipient nonfunctional yellow fluorescent protein (YFP)/blasticidin S deaminase (BSD) fusion genes, which enabled detection and selection of ectopic HR events by the restoration of YFP fluorescence and blasticidin S resistance by integration of the I-*SceI* gene (Arazoe et al. 2014) (Fig. 4). Furthermore, a translocated duplication or triplication of a targeted chromosomal region via I-*SceI*-induced break was developed in *A. oryzae,* which then showed significant increases in protease and amylase activities (Takahashi et al. 2018). The results suggested that gene-dosage effects were enhanced by segmental duplications of chromosomes. The precondition of the method is that I-*SceI* restriction sites must be inserted in advance at the changed sites, which increases the complexity of operation compared with using the CRISPR-Cas9 system. Therefore, it is not widely used in most filamentous fungi.

Gene expression system

Integration of target genes into the genome

The selected promoter and chromosomal integration sites are usually considered major factors affecting gene

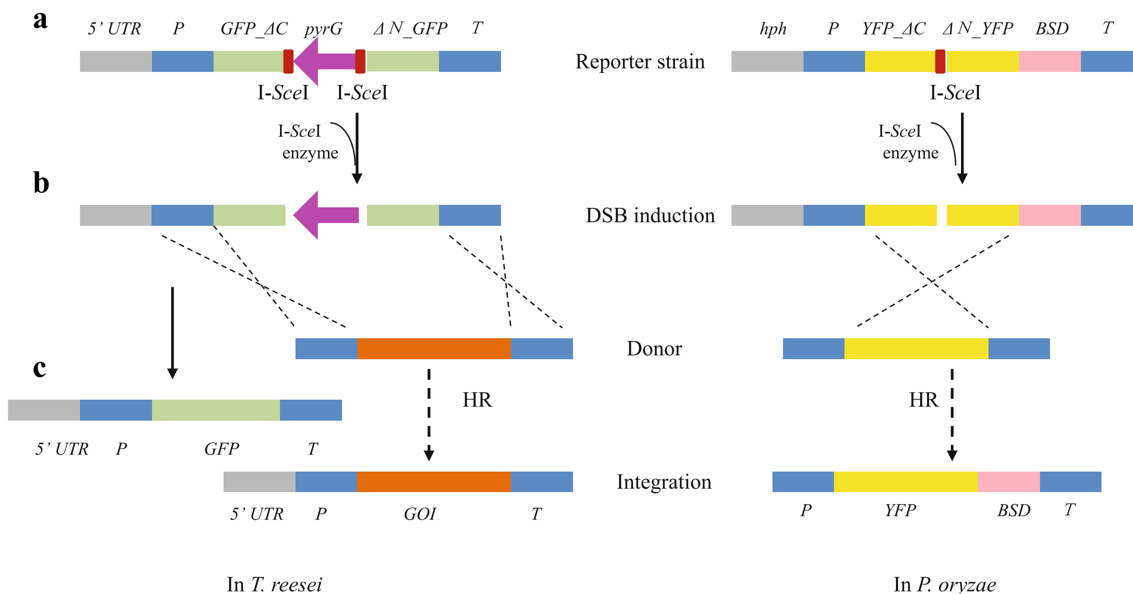

Fig. 4 The process of targeted integration of the GOI expression cassette via I-*SceI* enzyme-mediated homologous recombination in *T. reesei and P. oryzae* . a A reporter strain of *T. reesei* which was uridine auxotrophic and with nonfunctional GFP was engineered by inserting two I-*Sce*I restriction sites at the *cbh2* locus, producing a uridine prototrophic strain containing two I-*Sce*I sites surrounding the *pyrG* cassette, but in *P. oryzae*, this system consisted of donor and recipient nonfunctional yellow fluorescent protein (YFP)/blasticidin S deaminase (BSD) fusion genes. b Both the GOI-expressing cassette and I-*Sce*I enzyme were cotransformed into the reporter strain to create DSB and the expression of I-*Sce*I generated two DSBs and the loss of the *pyrG* selection marker between the I-*Sce*I sites. c The DSB could be repaired by homologous recombination with the GOI-expressing cassette or GFP which has homologous regions to the locus containing the I-SceI sites

expression. In the past decade, research has shown that highly expressed genes and their core promoters play important roles in the initiation of the transcription and expression of GOIs (Danino et al. 2015; Fitz et al. 2018). Cellobiohydrolases are the main proteins highly secreted by *T. reesei*. To express the lipase gene from *Talaromyces thermophilus* in *T. reesei*, a self-designed *CBH1* promoter was used to drive the lipase gene expression by flanking sequences of the *cbh1* gene using a binary vector for homologous recombination (Zhang and Xia 2017). This method can also transport secreted proteins to the extracellular space, which is very beneficial for the separation and purification of protein products. In addition to the *cbh1* gene locus, two other genome insertion loci, R3 (the 5′-*UTR* region of the cel3c gene) and R11 (transcription factor gene, 5 kb AT-rich sequences downstream of the insertion site), were identified that efficiently facilitated gene expression in *T. reesei* (Qin et al. 2018). Overexpression of the *cexA* transporter gene leads to a significant increase in citric acid secretion by integrating the *cexA* transporter gene into the locus *pyrG* using the strong constitutive *pmbfA* promoter and an inducible Tet-on expression system in *A. niger*, yielding up to 109 g/L citric acid, which is 3 times higher than the yield of the constitutive expression system (Steiger et al. 2018). Gene copy number polymorphisms depend on gene flanking regions. An engineered *A. oryzae* simultaneously producing cellobiohydrolase, endoglucanase, and β-glucosidase was constructed by integrating multiple copies of the corresponding genes into fungal chromosomes and showed approximately 10-fold higher activity than single integration strains (Wakai et al. 2018). A novel strategy of site-specific integration of foreign DNA via sulfonylurea resistance reconstitution efficiently replaced the native *ILV2* with the sulfonylurea-resistant *ILV2*(SUR) variant in *Magnaporthe oryzae*, which eliminated position/orientation effects and unnecessary mutations, in contrast to random ectopic integration (Yang and Naqvi 2014). In addition, an appropriate site and flexible integration strategy are important for the integration of target genes into the genome. A high integration efficiency into the fungal genome was achieved using Golden Gate cloning to facilitate the DNA construction process in *A. niger* (Sarkari et al. 2017), which provided high design flexibility for the integration of an expression cassette into the genome. A suitable genome site for the insertion of a target gene into the fungal genome is an effective strategy, as alternation between hyphal growth and the formation of uni- or multinuclear conidia makes it difficult to maintain a population of autonomously replicating plasmids carrying high copy numbers of the GOI. Therefore, suitable integration sites still need to be developed in the future.

Expression cassette construction depending on the host

Constructing the expression cassette to be the host-specific is a universal strategy. The main building blocks for alternative fusion strategies consist of (1) a gene expression promoter, a target gene, and a transcription terminator (such as cbh1or cbh2 or egl2) (Miyauchi et al. 2013); (2) a carrier-linker component (carrier includes the cbh1 core, man1 core and core-hinge, and cbh2 or egl2 core) (Su et al. 2012) and a linker region that is natural or synthetic to create space between different domains (Nevalainen et al. 2018); (3) a multicloning site (MCS) and a transformation selection marker; and (4) a purification tag such as 6×His or FLAG that can be incorporated into the MCS and a protein secretion signal. The expression constructs are thus modular in nature, allowing for addition, swapping, or exclusion of particular parts as required. The development of promoters plays a prominent role in filamentous fungi (Kluge et al. 2018). The identification of new and tunable promoters with different expression strengths was simplified with the development of genomics and transcriptomics. Regulatory elements used for protein expression show that natural constitutive promoters of glyceraldehyde-3-phosphate dehydrogenase (*gpd*), tryptophan synthase *(trp1*, *trpC*), translation elongation factor (*TEF*), and other protein families could be used to control gene expression in fungi such as *Cochliobolus heterostrophus*, *A. niger*, *A. nidulans*, the endophytic fungus *C. arbuscula,* and *Aspergillus terreus* (Sureka et al. 2014; Zhang et al. 2018a; Cao et al. 2018; Hasan et al. 2018). Inducible promoters are typically used to control the amount of gene expression under special conditions. For example, a benzoate-inducible promoter (*Pben*), a xylan-inducible promoter (*Pxyl*), an alcohol-inducible promoter ($AlcR\text{-}P_{alcA}$), and a sorbitol-induced promoter (*Psor*) can control gene expression in fungi such as *A. niger*, *A. nidulans*, *Chlamydomonas reinhardtii*, and *A. oryzae* (Antunes et al. 2016; Ma et al. 2018; Lee et al. 2018; Oda et al. 2016). *Pgas* is a low-pH-inducible promoter; *Pgas* strength was independent of the acid type and acid ion concentration, showing a dependence on pH only, and promoted gene expression at the low pH of 2.0 in *A. niger* producing itaconic acid at a titer of 4.92 g/l (Yin et al. 2017). Despite current advances in effective promoters, cassettes are typically based on a pUC-based vector for autonomous replication sequence elements. Autonomous replication sequence elements have been found in *F. oxysporum* (Powell and Kistler 1990), *A. nidulans* (Aleksenko and Clutterbuck 1997), *A. gossypii* (Schade et al. 2003), and *Phanerochaete chrysosporium* (Rao and Reddy 1984). The endoglucanase I and cellobiohydrolase I (CBHI) from *T. reesei* were successfully expressed in *A. gossypii* (Ribeiro et al. 2010), but these have not been widely harnessed for high-level gene expression (Chen et al. 2018). Construction of mutant strains is another way to

enhance gene expression. A *P. chrysogenum* Δ*paf* mutant strain acts as a microbial expression factory and lacks the *paf* gene coding for the endogenous antifungal protein so that it is resistant to antifungal proteins from other ascomycetes. Expression of the cassettes of recombinant proteins was driven by the strong *paf* promoter and the presence of a *paf*-specific sequence transporting cysteine-rich antifungal proteins in different *Penicillium* species into the supernatant (Garrigues et al. 2018; Sonderegger et al. 2016). In the expression cassette, reporter genes can be qualitatively or quantitatively analyzed via promoter or gene expression activities within the cell (Xiong et al. 2012); these reporter genes include 4′-phosphopantetheinyl transferase (*npgA*), luciferase (*luc*), β-glucuronidase (*gusA*), and β-galactosidase (*lacZ*) (Song et al. 2018a; Nordeen 1988; Bronstein et al. 1996; Matsumura et al. 1999). A GFP fusion-coupled fluorescence-activated cell sorting platform and a constitutive promoter were established to speed up the rapid selection of expressible heterologous genes (Wang et al. 2018a; March et al. 2003). L-Methionine-repressible promoters could be used to tune gene expression in *T. reesei* (Bischof et al. 2015). A cassette with a GFP-tagged vector was used to evaluate the biocontrol effects in the endophytic fungus *Acremonium implicatum* (Yao et al. 2015). These studies suggest whether a constructed cassette can be expressed depending on the specific filamentous fungal host. Most of these cassettes are not universal in other hosts. However, new hosts with excellent natural characteristics can provide favorable opportunities for detailed functional studies of different microorganisms and application research, and the expression systems of different organisms usually require endogenous gene expression regulatory elements to reduce interference. Therefore, a suitable expression cassette still needs to be developed and will pave the way for the application of new hosts.

Universal expression system independent of the host

Most expression systems depend on an individual host. Several reports have demonstrated that high gene expression depends on high levels of natural transcription regulators (McIsaac et al. 2014; Ottoz et al. 2014; Ito et al. 2015; Sadowski et al. 1988). Most of these systems are regulated by externally added compounds such as estradiol, testosterone, or doxycycline, which are often potential barriers due to cost and are not commercially viable. Although these systems are important tools, there is no evidence that they can control gene expression in other species (Blount et al. 2012; Belli et al. 1998). A synthetic expression system (SES) was developed that can be used in a broad spectrum of fungal species without host-dependent optimization or special genetic manipulation tools for special fungi (Rantasalo et al. 2018), as shown in Fig. 5. The SES consists of two parts: weak but constitutive expression of a designed transcription factor (*sTF*) (e.g., Bm3R1-NLS-VP16, Bm3R1 encoding the DNA-binding protein) and a strong *sTF*-dependent promoter that enables tunable expression of the target gene via changing the number of BSs to result in different expression levels of the target gene without using exogenous inducers. First, screening of proper promoters was needed. Expression of an *sTF* was achieved by a core promoter 1 (CP1) instead of a full-length promoter, which provided the input signal for constitutive expression of *sTF*. Then, the *sTF* recognized BSs upstream of the reporter gene synthetic promoter, which was designed to provide an output signal by varying the number of BSs and the selection of a core promoter 2 (CP2). The promoters and linearized centromeric plasmids were cotransformed into *S. cerevisiae*. The linearized centromeric plasmids contained BSs, a *LexA*-VP16 *sTF* (LexA encoding DNA-binding transcriptional repressor), an mCherry reporter gene and a *LEU2* selection marker (Fig. 5a). Second, the construction of universal cassettes was constructed. The cassettes of the input signal and output signal were integrated into the corresponding site to trigger expression of the GOI in different yeasts and filamentous fungi (Fig. 5b). The SES functionality has been used in six yeast and two filamentous fungus species, and has been used as well as to adjust the expression levels of heterologous and native genes. The SES is an unprecedentedly broadly functional gene expression regulation method. Importantly, it enables the use of novel eukaryotic microbes for basic research and various biotechnological applications.

Reported-guided gene expression

Promoter exchange can activate target gene, but it is only feasible in relatively simple systems consisting of a few operons. In addition, this method requires an advanced molecular genetic manipulation system to manipulate chromosomal DNA, which may not be available for every microorganism. Reported-guided gene expression about Tet-off or Tet-on system has been used to overexpress for target genes and activate targeted activation of silent gene clusters as an efficient strategy (Das et al. 2016; Bijlani et al. 2018), as shown in Fig. 6. The initial Tet-induced regulatory gene expression system was established based on the Tet-resistant operons of the Tn10 transposon in *E.coli* (Bertram and Hillen 2008; Meier et al. 1988; Hillen and Berens 1994). The Tet-off gene regulatory expression system consists of a regulatory expression vector and a response expression vector. The regulatory system contains a promoter and tetracycline transcription activator (*tTA*). The *tTA* is fused using *TetR* with a transcriptional activation region at the C-terminal of herpes simplex virus VP16. The response system is composed of a Tet-responsive element (TRE) with seven repetitive *TetO* sequences, a minimal promoter (*Pmin*) lacking enhancers, and a target gene. When Tet or Dox exist, they can change the conformation of *TetR* in *tTA*, and *tTA* will fall off from TRE and inactivate Pmin, stopping

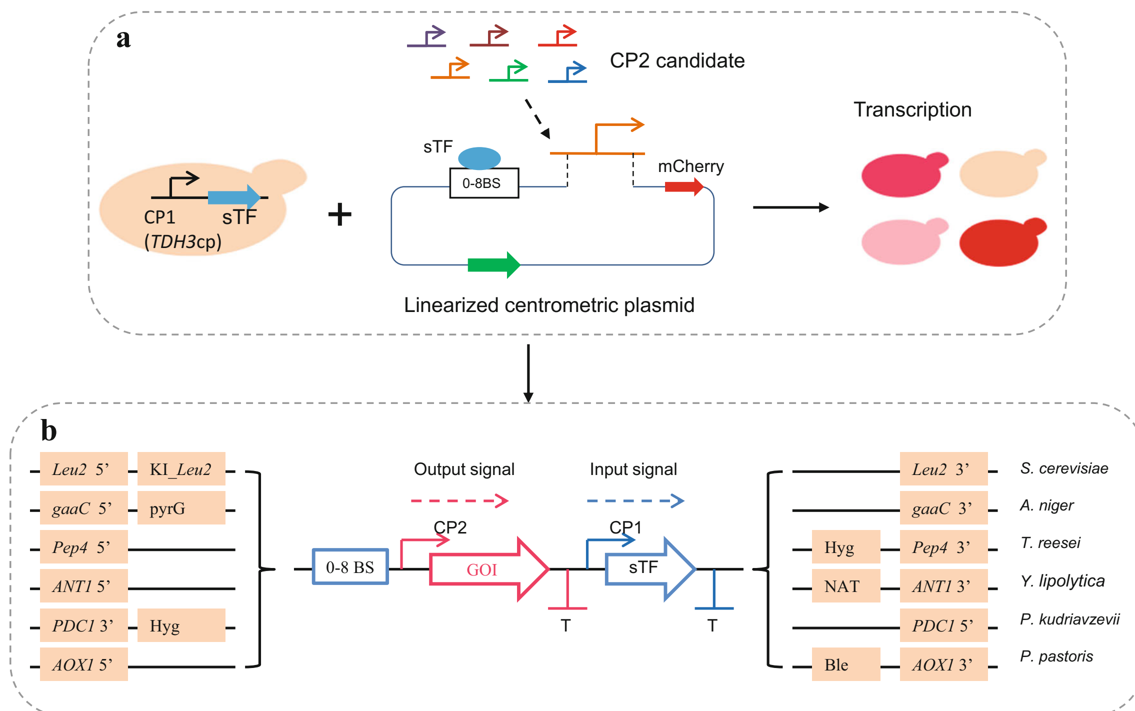

Fig. 5 Principle of the synthetic expression system based on transcription amplification. a Screening of promoters. Expression of an *sTF* was achieved by a CP1 promoter (e.g., *TDH3* promoter), which provided constitutive expression as the input signal and the *sTF* recognized BS in the upstream of the report gene synthetic promoter, which was designed to provide the output signal by varying the number of BSs and selection of a CP2. The CP2 were assembled with a linearized centromeric plasmid in vivo (cotransformed) in a *S. cerevisiae* strain expressing the *LexA*-VP16 *sTF* (and in parallel in an isogenic strain lacking the *sTF*). Transcription amplification that represented the capacity of the CP to trigger transcription initiation in the presence and absence of the sTF was assessed by the expression of report gene. b Construction of universe cassettes. The cassette of the input signal and output signal was integrated into the corresponding site to trigger expression of the GOI in different yeast and filamentous fungi (*Leu2* encoding 3-isopropylmalate dehydrogenase in *S. cerevisiae*, *gaaC* encoding 2-keto-3-deoxy-L-galactonate aldolase in *A. niger*, *Pep4* encoding in *T.reesei*, *ANT1* encoding adenine nucleotide translocator in *Y. lipolytica*, *PDC1* encoding indolepyruvate decarboxylase 1 in *P. kudriavzevii*, *AOX1* encoding aldehyde oxidase 1 in *P. pastoris*)

gene expression (Fig. 6a). When there is no Tet or derivative Dox in cells, tTA can bind to TRE and result in gene expression (Fig. 6b). The difference between the Tet-on regulatory system and Tet-off regulatory system is that its regulatory protein is reverse tetracycline transcription activator (*rtTA*). *RtTA* is a fusion of reverse *TetR* and the VP16 transcriptional activation region. The *rtTA* is derived from the mutation of four amino acids in *TetR* (E19G and A56P were shown to reverse the phenotype whereas the other amino acid mutations D148E and H179R are involved in effector binding and dimerization) (Urlinger et al. 2000). The phenotype of *rtTA* is contrary to that of *tTA*. Without Dox, *rtTA* cannot bind to TRE, which results in gene expression shutdown (Fig. 6c). In the presence of Dox, rTetR binds to TRE, leading to gene expression (Fig. 6d). The tetracycline-induced expression system has been used in *A. niger* by adding Dox (Meyer et al. 2011; Berens and Hillen 2003; Baron et al. 1997). Tet-On expression cassettes resulted in the formation of one long polycistronic mRNA by adding doxycycline as an inducer. The system can be used to express at least three genes polycistronically in *A. niger* (Schuetze and Meyer 2017). The Tet-On System was also used for the activation and expression of the trichosetin gene cluster in *Fusarium fujikuroi* (Janevska et al. 2017). In addition, the luciferase reporter gene was simultaneously used to test the quantification of gene oscillations in *A. niger* (Wanka et al. 2016). Tet-induced expression systems have low disturbance and high levels of induction. The induction time is short and the gene expression can be detected in 30 min after adding the inducer. The system is reversible and it can be shut down after eliminating the inducer and can be reopened after adding the inducer. Tet and its derivative Dox are relatively safe drugs. This strategy is suitable to the research for secondary metabolites, but not for interaction between endophytic fungi and host. In addition, a copper-mediated gene expression on-off system based on the copper-responsive promoter Ptcu1c from *T. reesei* was also developed (Wang et al. 2017b). Maybe, it was useful to precisely manipulate some biological processes in filamentous fungi.

CRISPRi and CRISPRa

The newly developed CRISPR interference (CRISPRi) and CRISPR activity (CRISPRa) systems were developed to

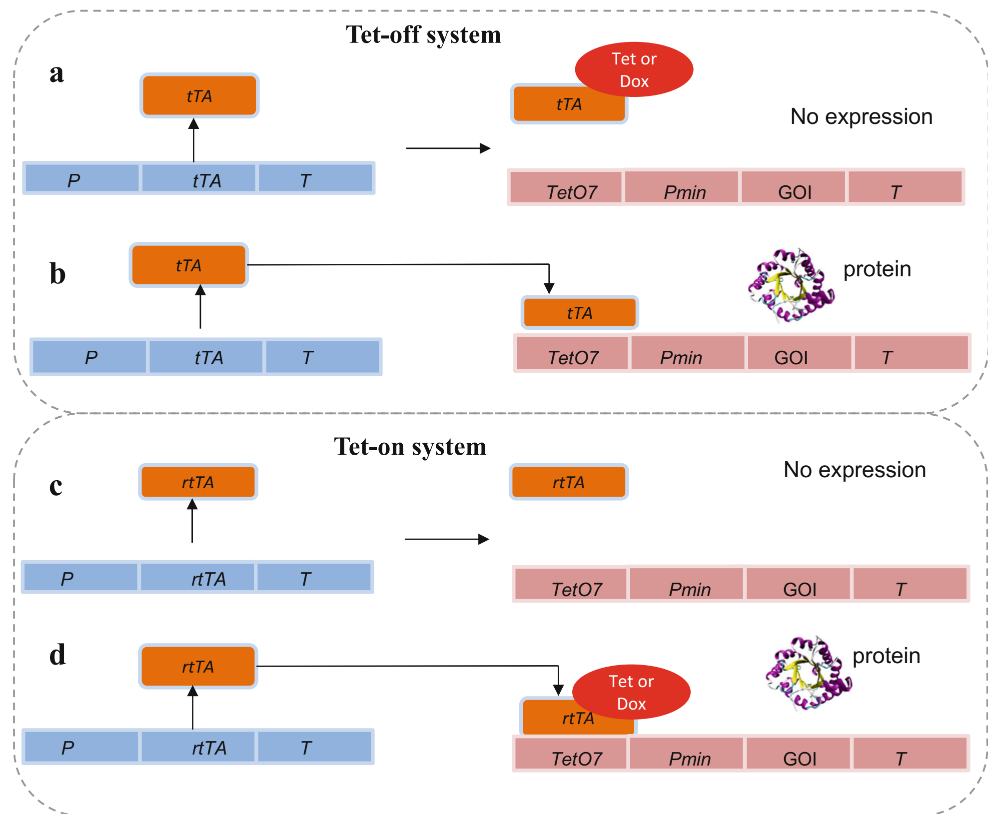

Fig. 6 Principle of the Tet-on system depending on the reverse tetracycline-controlled transactivator (*rtTA*) and Tet-off system depending on the tetracycline-controlled transactivator (*tTA*). a Gene transcription is reversibly turned off when Dox is added in the Tet-off system because of the Dox-induced separation of *tTA* from *tetO7*. b Gene transcription is reversibly turned on in the absence of Dox in the Tet-off system because of the Dox-induced combination of the *tTA* protein with *tetO7*. c Gene transcription was turned off in the absence of Dox, so that the expressed *rtTA* protein cannot combine to its operator binding site *tetO7* in the Tet-on system. d Gene transcription was reversibly turned on by the addition of Dox in the Tet-on system, so that the expressed *rtTA*-inducing association of the *rtTA* protein to its operator binding site *tetO7* and the GOI can be expressed.

regulate gene expression. A nuclease-dead Cas9 (dCas9) paired with a gRNA that targeted different regions of the promoter of an endogenous gene resulted in repressed transcriptional repression. Furthermore, the fusion of dCas9 to an Mxi1 repressor domain enhanced transcriptional repression in *C. albicans* (Wensing et al. 2019). A graded expression method was established via dCas9 regulation by varying the sgRNA target location to recruit dCas9 activator/repressor in promoters, producing a ~ 40-fold gene expression (Deaner and Alper 2017). Román et al. used fusions between a dCas9 and specific repressors (Nrg1) or activators (Gal4) that which resulted in specific repression or activation of the cytosolic catalase (Román et al. 2019). It is important to enable partial loss of gene function by repression or activation rather than a complete loss of function. CRISPRi and CRISPRa are attractive methods for use in filamentous fungi in the future.

Selection markers

Selection markers are very important for gene knockout and gene expression. Usually, nutrition-deficient markers and resistant markers are the main screening markers. There are two kinds of nutritional screening. The first is for nitrogen and carbon nutritional genes, such as the *amdS* gene (*A niger* grew poorly on acetamide as a nitrogen, so that it could be developed a selecting system for *A. niger* using the *amdS* gene encoding the acetamidase as a dominant heterologous marker and then degrading acetamide into ammonia to obtain the nitrogen source needed for its growth) (Kelly and Hynes 1985; Michielse et al. 2004), the *niaD* gene (nitrate reductase gene, *niaD*-deficient mutants can tolerate high concentrations of chlorate compared with the wild-type) (Whitehead et al. 1990; Navarrete et al. 2009; Ishi et al. 2005), and *glmS* genes (glucosamine synthase gene, *glmS*-knockout mutants can grow normally only on medium with exogenous glucosamine as carbon source) (Ram et al. 2004). The second type of screening is for complementary nutrition-deficient genes such as *pyrG* or *ura3* (encoding the glycoside-5′-phosphate decarboxylase gene) (Kumakura et al. 2019), *TRP* (encoding the tryptophan synthase gene) (Bruni et al. 2018; Kim and Marzluf 1988), *ArgA* (encoding the arginase gene) (Dave et al. 2012) or *ArgB* (encoding acetylglutamate kinase) (Jin et al. 2004a), and *HisB* (encoding the histidine synthase gene) (Fiedler et al. 2017). They cause the corresponding deficient mutants to only grow on the medium supplemented with uridine or uracil, tryptophan, arginine, or histidine, respectively. The nutrition-deficient markers have been widely used in filamentous fungi (Matsu-Ura et al. 2015; Fiedler et al. 2017; Jin et al. 2004a, b).

At present, the most commonly used resistance screening marker genes are antibiotic resistance genes. The inhibitory effects of antibiotics on filamentous fungi mainly lie in the formation of the cell wall, the function of the cell membrane, and the inhibition of the biosynthesis of nucleic acids or

proteins (Debono and Gordee 1994). The common antibiotic genes include *hygR* (encoding the hygromycin phosphotransferase gene) (Wang et al. 1988), *nat*1 (encoding nourseothricin acetyltransferase), *neoR* (encoding aminoglycoside phosphotransferase) (Liu et al. 2017), *bleR* (encoding the N-acetyltransferase gene) (Suzuki et al. 2009), and *BarR* (encoding phosphatidylinositol acetyltransferase) (Arazoe et al. 2015). The different acceptor fungi usually show different sensitivity and tolerance to antibiotics. Therefore, it is very important to determine the appropriate antibiotics and concentration of antibiotics for screening transformants. Scientists generally believe that phosphinothricin is an ideal screening marker for genetic transformation of filamentous fungi. Phosphinothricin is a broad-spectrum herbicide with almost no soil residue and is widely used in agricultural production due to its low price (Christ et al. 2017). These selection markers are used under selection pressure, which can be easily generated in the laboratory but is difficult to be used for natural condition, especially for the research between endophytes and plants. Therefore, screening markers are essential for genetic manipulation in most filamentous fungi. Nutrition screening markers are more economical than drug screening markers, but the acquisition of auxotrophic strains and high-frequency reversion mutation rates are a problem. Drug screening markers are simpler and more convenient, but not all antibiotics can be used for fungi. In addition, the high cost and safety of antibiotics cannot be ignored: the lateral transfer of genes makes endowed filamentous fungi resistant, which poses an ecological hazard to the living environment of humans.

Conclusion

Filamentous fungi are widely used in industrial, agricultural, and pharmaceutical production. Recent studies have found that endophytic fungi and their secondary metabolites play significant roles in synthesizing metabolites and promoting plant growth, disease resistance, and stress resistance. Therefore, the development of genetic manipulation systems for filamentous fungi will lay the foundation for the study of new secondary metabolites and the interaction between endophytes and plants. To this day, most of the genetic tools are about model strains, especially, endophytic fungi are less considerated. We suggest that the genetic manipulation tools for endophytic fungi should be two sets of system, one is for the study of secondary metabolites and the other is for the interaction between fungi and host. The resistant and auxotroph selection system may be suitable for the study of secondary metabolites, but the resistant and auxotroph selection system may be not suitable for the study interaction between endophytic fungi and its host plant. Because resistant marker may affect the growth and physiology of the plant, auxotroph selection markers are not easy to be controlled when endophytic fungi colonizing in the plant. Therefore, scarless knockout or recyclable screening markers will be helpful for more fungi in the future. Suitable screening markers are still challenged, especially for multiple gene editing, and not all screening markers are efficient for fungi. Novel promoters and universal plasmids are limited for a new host. It will be important to develop promoters and universal expression systems for novel hosts because multigene coexpression will depend on the development of promoters and expression systems. Synthetic expression systems were only developed in six fungi without host-dependent optimization (Rantasalo et al. 2018). This may be a start to the development of a universal expression system that can be widely used in novel filamentous fungi. Reported-guided gene expression was an efficient strategy that overcomes the shortcomings of the above methods such as screening of strong promoters and promoter exchanges. Knowledge of the secondary metabolites of filamentous fungi and the interactions with plants will break through the current bottleneck and usher in new development opportunities.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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