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MINI-REVIEW

Molecular tools for gene manipulation in filamentous fungi

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Abstract Functional genomics of filamentous fungi has gradually uncovered gene information for constructing 'cell factories' and controlling pathogens. Available gene manipulation methods of filamentous fungi include random integration methods, gene targeting technology, gene editing with artificial nucleases and RNA technology. This review describes random gene integration constructed by restriction enzyme-mediated integration (REMI); *Agrobacterium*-mediated transformation (AMT); transposon-arrayed gene knockout (TAGKO); gene targeting technology, mainly about homologous recombination; and modern gene editing strategies containing transcription activator-like effector nucleases (TALENs) and a clustered regularly interspaced short palindromic repeat/associated protein system (CRISPR/Cas) developed in filamentous fungi and RNA technology including RNA interference (RNAi) and ribozymes. This review describes historical and modern gene manipulation methods in filamentous fungi and presents the molecular tools available to researchers investigating filamentous fungi. The biggest difference of this review from the previous ones is the addition of successful application and details of the promising gene editing tool CRISPR/Cas9 system in filamentous fungi.

Keywords Filamentous fungi · Molecular tools · Gene manipulation · CRISPR/Cas9

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Introduction

Filamentous fungi are of special interest to researchers because of their great capacity to produce diverse valuable metabolites, including antibiotics (Meyer et al. 2011), enzymes (Hoffmeister and Keller 2007), acids (Magnuson and Lasure 2004) and lipids (Tang et al. 2016) (Chen et al. 2015), in addition to detrimental toxins such as aflatoxins (Sørensen et al. 2008) and sterigmatocystins (Keller and Hohn 1997). Molecular tools are needed to manipulate them, such as strengthening biosynthetic pathway to construct microbiological cell factories and disrupting pathogenic genes to control the harmful filamentous fungi. Besides, genome sequences of hundreds of filamentous fungi have been established (http://www.genomesonline.org/; http://genome.jgi.doe.gov/), and effective gene manipulation techniques, especially multiple-gene and target-specific methods, are needed for exploration of genetic information to promote its application in the pharmaceutical field, agricultural field and food industry.

Various transformation methods of filamentous fungi, including protoplast transformation (Case et al. 1979, Turgeon et al. 2010), particle bombardment (Bhairi and Staples 1992), electroporation (Richey et al. 1989) and *Agrobacterium* -mediated transformation methods (de Groot et al. 1998a) presented in Table 1 (Meyer 2008), have been established. And multiple selection markers summarised in Table 2, (referring to

Table 1 Four common strategies for transformation of filamentous fungi

Methods	Principles	Advantages	Disadvantages
PMT	Use cell wall-degrading enzymes to prepare protoplasts	Spores, germlings and hyphal tissue can be used	Transformation rate depends on the particular batch of lytic enzyme
	Uptake the DNA by the addition of PEG and $CaCl_2$		Requires regeneration procedure
			High copy number of inserted DNA
AMT	*A. tumefaciens* carries two vectors (the binary vector with the DNA of interest between the left and right border repeats and the T-vector containing the virulence region important for DNA transfer)	Spores, germlings and hyphal tissue can be used	Various parameters during co-cultivation affect transformation rate
	DNA transfer is achieved during co-cultivation of *A. tumefaciens* with the fungus	Low copy number of inserted DNA Improves targeted integration	More time-consuming
EP	Uptake DNA is mediated by reversible membrane permeabilisation induced with local application of electric pulses	Spores and germlings can be used Simple and cheap	Protoplast formation is often needed
BT	DNA are coated with tungsten or gold and accelerated into cells at high velocity	Does not require pre-treatment of recipient cells	Requires special equipment

Adapted from *Genetic engineering of filamentous fungi-Progress, obstacles and future trends. Doi:* https://doi.org/10.1016/j.biotechadv.2007.12.001

PMT protoplast-mediated transformation, *AMT Agrobacterium*-mediated transformation, *EP* electroporation, *BT* biolistic transformation

(Fincham 1989)), such as hygromycin B drug resistance (Kück et al. 1989; Punt and van den Hondel 1992) and uracil auxotrophic mutants (Ando et al. 2009; Hao et al. 2014) are available. Those all facilitate the progress of the genome manipulation method in filamentous fungi with addition of the development of corresponding screening systems.

The random integration method is applied for high-throughput mutagenesis in a strain of interest, but its process is tedious. Gene targeting technology based on the homologous recombination method can help us manipulate particular genes but this method is not efficient in filamentous fungi. Gene editing technology appears after the genome sequencing can be used for efficiently uncovering gene function, and one of the most promising tools—CRISPR systems—was successfully used in filamentous fungi. The RNA technology can disturb gene expression in the translational level, but is not applicable in some filamentous fungi. Those molecular tools in filamentous fungi allow researchers to choose proper methods for uncovering gene function and manipulating different genes efficiently.

This review provides an insight into the gene manipulation methods applied in functional genomics in filamentous fungi and details up-to-date gene editing strategies, with a focus on the CRISPR/Cas9 system.

Random integration methods

Gene functions have traditionally been tested with random gene mutation methods using physical mutagens, including ionising radiation, ultraviolet light or radioactive chemicals and chemical mutagens such as alkaloids and benzene (Casselton and Zolan 2002) to determine whether any phenotypic change had occurred. However, the process of isolation and recovery of the mutated gene is tedious. The detection of

Table 2 Common selection markers used for transformation of filamentous fungi

Marker	Phenotype	Case reported	Disadvantages
Drug resistance	Hygromycin B resistance	*Cephalosporium*	Selection difficulties existed due to weak dominance resistance over wild-type allele
		Fulvia	
	Benomyl resistance	*Neurospora crassa*	
	Oligomycin resistance	*Aspergillus niger*	
Auxotrophic markers	Acetamide utilisation	*Aspergillus nidulans*	Difficult to achieve in species without proper gene genetic systems
	Pyrimidine synthesis	*Neurospora crassa*	
	Arginine synthesis	*Aspergillus nidulans*	
Visible markers	β-galactosidase	*Aspergillus*	Specific chemicals or instruments are needed

genetic mutations with molecular tools saves the laborious work involved in mutation isolation and gene identification.

Random gene disruptions can be created through transformation methods (foreign DNA insertion) or by the movement of mobile genetic elements. Other related gene manipulation methods used in filamentous fungi are restriction enzyme-mediated integration (REMI) and transposon-arrayed gene knockout (TAGKO) (Jiang et al. 2013).

Restriction enzyme-mediated integration

REMI was first established in 1991 in *Saccharomyces cerevisiae* (Schiestl and Petes 1991). The basic process of REMI transformation and recovery of mutated genes is demonstrated in Fig. 1 (Kahmann and Basse 1999). After the enzyme-digested DNA fragment and the enzyme are transformed into the protoplast, the plasmid can be integrated into the genome of the host based on the same sticky ends. The mutated genes can be isolated with their corresponding primers after plasmid excision and ligation after transformation in *Escherichia coli.* REMI has been successfully used in filamentous fungi, mainly for the identification of pathogenicity genes. One example is the filamentous ascomycete *Magnaporthe grisea*. Shi et al. screened 10 mutants for sporulation, pathogenicity and auxotrophy among 800 transformants with the REMI method (Shi and Leung 1995). Sweigard et al. also used this method to achieve 27 stable pathogenic mutants among 5538 transformants in *M. grisea* (Sweigard et al. 1998). Researchers have also found that the use of enzymes in the REMI method improves transformation efficiency (Shi et al. 1995). In addition, this method has already been used in the filamentous fungi *Monacrosporium sphaeroides* (Xu et al. 2005), *Fusarium oxysporum* (Imazaki et al. 2007) and *Pleurotus eryngii* (Noh et al. 2010); approximately 5000 transformants and 2929 transformants were achieved for the first two species, respectively, and 10 to 40 transformants per 10^6 protoplasts were achieved for the third.

Several disadvantages hold back the development of this method. It was initially dependent on protoplasts for the uptake of plasmid DNA and enzymes. The proper choices and concentrations of enzymes must be determined before the transformation. Researchers also found that different restriction enzymes vary significantly in their ability to integrate fragments into the host genome (Manivasakam and Schiestl 1998). If plasmid rescue is needed, essential enzymatic sites must be known.

Agrobacterium-mediated transformation

The *Agrobacterium*-mediated transformation (AMT) method was traditionally used in plants (Valvekens et al. 1988; Tingay et al. 1997; Komari et al. 1996) and was later applied to yeast (Piers et al. 1996), filamentous fungi (de Groot et al. 1998b) and mammal cells (Kunik et al. 2001). *A. tumefacians* is a gram-negative plant pathogen that can transfer its T-DNA randomly into genome of the recipient at a random site (Hooykaas and Beijersbergen 1994) upon induction via chemicals, usually acetosyringone. Acetosyringone was used as induction through activating VirA and VirG proteins on the surface of *Agrobacterium tumefaciens* to send signals. T-DNA is bordered by 25 base pair repeats on each end. And the transfer is initiated at the right border and terminated at the left border in presence of the Vir proteins. The specific AMT process (Michielse et al. 2005) is illustrated in Fig. 2.

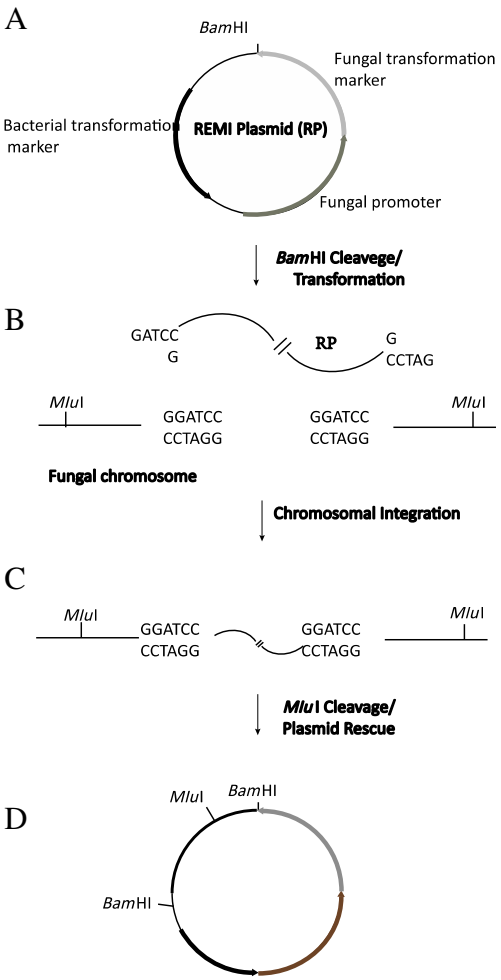

Fig. 1 REMI transformation and plasmid rescue. Adapted from *REMI (Restriction Enzyme Mediated Integration) and its Impact on the Isolation of Pathogenicity Genes in Fungi Attacking Plants. Doi*:https://doi.org/10.1023/A:1008757414036. **a** A REMI plasmid (RP) often contains a fungal transformation marker for gene transcription and a bacterial transformation marker for plasmid rescue. **b** The circular or linearised REMI plasmid is transformed into fungal cells together with a restriction enzyme (e.g. *Bam*HI), resulting in two cleavage sites at the transforming plasmid and chromosomal. **c** Free ends of the two cleaved sites are joined together. **d** Plasmid rescue can be achieved via plasmid excising from genome in presence of flanking sequences (e.g. by using *Mlu*I) and circularising with DNA ligase before transformation in *E. coli*

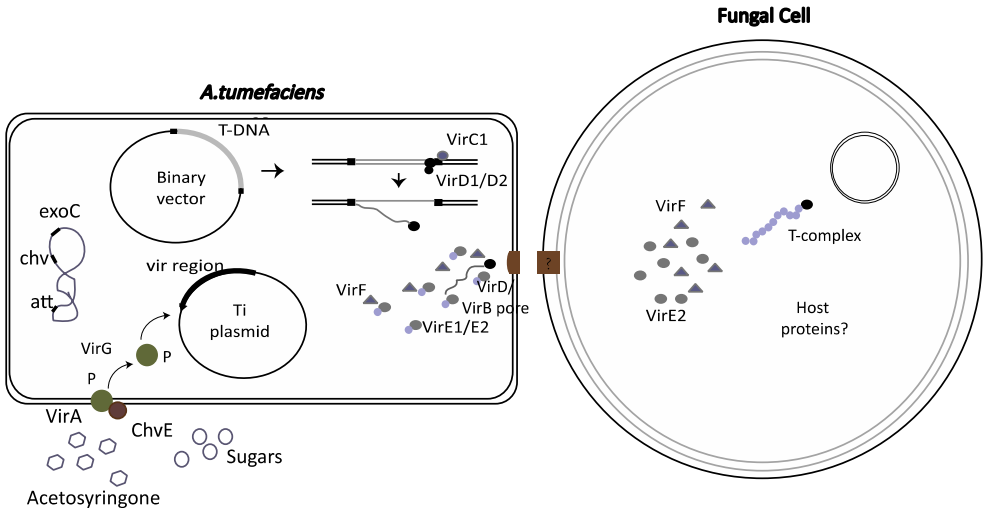

Fig. 2 Schematic overview of the *Agrobacterium tumefaciens* T-DNA transfer system. Adapted from *Agrobacterium-mediated transformation as a tool for functional genomics in fungi. Doi:*https://doi.org/10.1007/s00294-005-0578-0. When acetosyringone or sugars exists, the *vir* genes encoding the T-DNA transfer machinery of *A. tumefaciens* are induced. Virulence proteins VirA and VirG are activated first in the presence of acetosyringone, while chromosomally encoded protein, ChvE, interacts with the VirA protein upon recognition of monosaccharides. The VirG protein is activated through accepting the phosphoryl group from the activated VirA proteins. The activated VirG proteins have DNA-binding properties, and acts as a transcriptional activator of itself and other virulence genes located in the virulence region. The VirD2 protein, assisted by VirD1, nick precisely in the bottom strand of both border repeats, leading to T-DNA release. VirC1 can bind the 'overdrive' of T-DNA and help T-strand production. After the T-DNA is released, the T-DNA strand will transfer through the bacterial membrane and fungal cell wall via a type IV secretion mechanism. The VirB protein forms a transport pore. The inner membrane VirD assists in the transferring process. The virulence proteins VirF, VirE1/E2 are also exported via the pore. The transferred T-DNA is targeted to the nucleus and randomly integrated into the genome. The precise mechanism of T-DNA integration is not known

Combier et al. transformed the mycelium of *Hebeloma cylindrosporum* with this method; Southern blot analysis of 83 randomly selected transformants showed a unique plasmid insert pattern, and 60% was composed of single-transferred DNA copies. The left and right borders can achieve 85 and 15% of transformants, respectively, via the thermal asymmetrical interlaced polymerase chain reaction (TAIL-PCR) (Combier et al. 2003). TAIL-PCR was used for recovering unknown (mutated) gene adjacent to known sequences, such as the inserted T-DNA and transposon, through utilising nested known-specific primers with a high melting temperature (Tm > 65 °C) in consecutive reactions together with a short (15–16 nucleotides) arbitrary degenerate (AD) primer with a low Tm (about 45 °C) and 64–256-folds of degeneracy (Liu and Chen 2007). The relative amplification efficiencies of target and nontarget products can be thermally controlled; thus, the mutated gene can be amplified. Gento et al. also used the ATMT method in *Colletotrichum lagenarium* and obtained 150 to 300 transformants per 10^6 conidia, and highly efficient gene recovery was achieved via the thermal asymmetrical interlaced polymerase chain reaction (Tsuji et al. 2003).

When the technique is used in filamentous fungi, host cells can include protoplasts (Liu et al. 2015), spores (Hao et al. 2014; Wang and Li 2008), hyphae and sporocarps. This alleviates the need for protoplast isolation as required by REMI. Rogers et al. adopted both REMI and ATMT to identify pathogenic mutants for *Coniothyrium minitans* and found that 32 transformants (μg DNA^{-1}) were achieved with REMI while 37.8 transformants (5×10^5 $germlings^{-1}$) were achieved with ATMT. And single-copy DNA integrations occurred in 8% of REMI and 40% of ATMT transformants (Rogers et al. 2004). Although ATMT surpasses REMI in some aspects, it also has some clear disadvantages. ATMT is time consuming, and many of the parameters involved, such as the ratio of host cells to *Agrobacterium*, the co-cultivation temperature and the co-cultivation time, must be optimised to achieve a high frequency of transformation.

Transposon-arrayed gene knockout

Transposable elements are diverse and ubiquitous in major groups of filamentous fungi, including *Ascomycetes*, *Zygomycetes* and *Basidiomycetes*. They are used for gene mutation due to their ability to transfer among the host cells (Daboussi 1997).

The transposable-arrayed gene knockout technique was first used for high-throughput mutagenesis in *M. grisea*. The process is illustrated in Fig. 3 (Hamer et al. 2001). The transposon-mediated mutagenesis method has also been established in some other filamentous fungi. *Aspergillus niger* was found to harbour a non-autonomous transposon named *Vader*, for which an excision frequency of 1 in 2.2×10^5 was

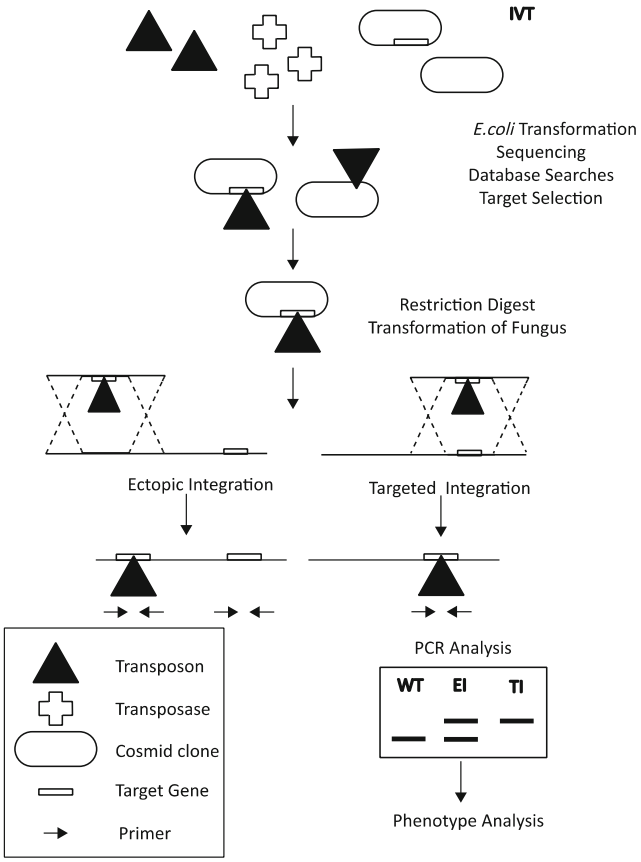

Fig. 3 Transposon-arrayed gene knockout. Adapted from *Gene discovery and gene function assignment in filamentous fungi. Doi:*https://doi.org/10.1073/pnas.091094198. An IVT reaction needs transposons, corresponding transposase and recipient DNA, usually a cosmid vector. Then, transform the IVT products into *E. coli*. Positive transposon insertion sites can be determined by sequencing. Then, the proper cosmids are digested with the homing endonuclease to release full-length inserts for transformation. Ectopic and TI events are distinguished by PCR analysis, and mutants are subjected to phenotype analysis. *IVT* in vitro transposition, *WT* wild type, *EI* ectopic integration, *TI* targeted integration

found (Hihlal et al. 2011). A similar method was used in *Penicillium griseoroseum* to achieve a transformation frequency based on a heterologous transposon, *Impala*, from *F. oxysporum* (de Queiroz and Daboussi 2003), and similar methods were established in other filamentous fungi, including *Mycosphaerella graminicola* (Adachi et al. 2002) and *Aspergillus fumigatus* (Firon et al. 2003). Although this method can be used interchangeably and requires a high-efficiency transformation approach (Jiang et al. 2013), target manipulation cannot be achieved.

Gene targeting technology

The functions of specific genes can be verified by gene overexpression and downregulation methods through homologous recombination.

Homologous recombination

The homologous recombination (HR) method was conventionally used for target integration for gene knock-in and gene mutation, but it was not as effective in filamentous fungi as in yeast. In *S. cerevisiae*, 50 bp was sufficient for foreign DNA integration (Hua et al. 1997). In filamentous fungi, larger fragments longer than 1000 bp may not achieve high transformation efficiencies (Kupfer et al. 1997). Although the frequency of homologous recombination of filamentous fungi can be improved by mutating the genes involved in the non-homologous end-joining process, usually *ku70* and *ku80*, the technique still depends upon protoplasts and a proper plasmid with a proper selection marker. Digestion of cell wall of filamentous fungi is usually difficult.

Gene editing technology

"Cre/*Loxp*" and "FLP/*FRT*" system

Before appearance of the concept of gene editing, there exists a selection marker recycle method called the "Cre/*Loxp*" system, allowing multiple genes manipulation in filamentous fungi. The "Cre/*Loxp*" system was adapted from P1 bacteriophage and was composed with a recombinase named Cre and two corresponding consequent *Loxp* sites. And the Cre can digest at the two consequent *Loxp* sites, resulting in a gene, usually a selection marker, within the two consequent *Loxp* sites being deleted. The Cre/Loxp system was applied in *Aspergillus* (Krappmann S et al. 2005, Forment et al. 2006, Mizutani et al. 2012, Huang et al. 2016), *Trichoderma* (Steiger et al. 2011), *Neurospora* (Honda and Selker 2009), *Neotyphodium* (Florea et al. 2009) and so on (Zhang et al. 2013, Aguiar et al. 2014) (summarised in Table 3).

Similarly, a "FLP/*FRT*" system, composed of FLP recombinase with corresponding *FRT* sites, is also used for marker recycling in filamentous fungi, but is derived from the yeast *S. cerevisiae*. Kopke et al. firstly demonstrated successful application of the "FLP/*FRT*" system in *Penicillium chrysogenum* and *Sordaria macrospora* in 2010 (Kopke et al. 2010). Later, this method was used in *Ustilago maydis* (Khrunyk et al. 2013) and *Acremonium chrysogenum* (Bloemendal et al. 2014).

The above two methods offer possibilities for multigene manipulation but need two steps to achieve gene recycle. Take the "Cre/*Loxp*" system for example, the *Loxp* sites as well as a marker gene need to be integrated into the host genome first, then the Cre needs expression to finish the gene deletion process. Thus, a precise selection process of the second step is necessary for successful application of the two methods.

Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat/associated systems (CRISPR/Cas) are gene manipulation systems that use

Table 3 Filamentous fungi with Cre/*Loxp* and FLP/*FRT* systems established

Recombinase	Filamentous fungi	Reference
Cre/*Loxp* from P1 bacteriophage	*Aspergillus fumigatus*	(Krappmann S et al. 2005)
	Aspergillus nidulans	(Forment et al. 2006)
	Neotyphodium coenophialum *Neotyphodium uncinatum*	(Florea et al. 2009)
	Epichloë festucae	
	Neurospora Crassa	(Honda and Selker 2009)
	Trichoderma reesei	(Steiger et al. 2011)
	Aspergillus oryzea	(Mizutani et al. 2012)
	Cryphonectria parasitica *Metarhizium robertsii*	(Zhang et al. 2013)
	Ashbya gossypii	(Aguiar et al. 2014)
	Aspergillus terrus	(Huang et al. 2016)
FLP/*FRT* from *Saccharomyces cerevisiae*	*Penicillium chrysogenum* *Sordaria macrospora*	(Kopke et al. 2010)
	Ustilago maydis	(Khrunyk et al. 2013)
	Acremonium chrysogenum	(Bloemendal et al. 2014)

nucleases. CRISPR/Cas9 is the most widely used. Although the ZFN method was established earlier than TALENs and CRISPR/Cas9 and has been applied to gene editing in plants and animals, we were unable to find any examples of its use in filamentous fungi. Therefore, we focus on TALENs and CRISPR/Cas9.

Transcription activator-like effector nucleases

Transcription activator-like effectors were first found in 2009 in the plant pathogen *Xanthomonas* and contain a central region of tandem direct repeats with 34 amino acids (Boch et al. 2009). Figure 4 is a simple illustration of the transcription activator-like effector nuclease (TALEN) method. Two specific amino acids, often the 12th and 13th and known as repeat-variable di-residues, are two hypervariable amino acids that determine the single nucleotide in the target DNA sequence (Christian et al. 2010). The artificial TALENs are fused with a DNA catalytic part *Fok*I to produce a specific double-strand break. Compared with the three bases recognised by one ZFN monomer, one TALEN monomer recognises one base and therefore offers a larger, more flexible target range (Sun and Zhao 2013).

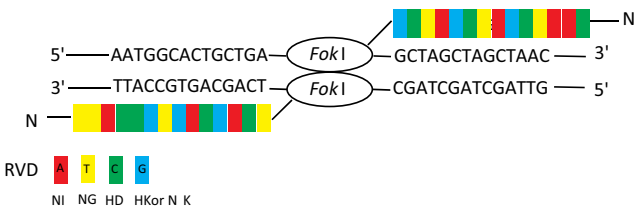

Fig. 4 Transcription activator-like effector nuclease (TALEN) in complex with target DNA. TALEN target sites contain two TALE binding sites with a spacer sequence separation of varying lengths (12–20 bp). Each TALE repeats recognise the specific base pair through two hypervariable residues and then linked *Fok*I cleavage at target site. *RVD* repeat-variable di-residue

This technology was first used in filamentous fungi in the rice blast fungus *Pyricularia oryzae* in 2015 (Arazoe et al. 2015), and the established platinum-fungal TALEN system raised the targeted gene replacement efficiency through homologous recombination by as much as 100%. This technique has not yet been reported in any other genus of filamentous fungi.

The technical challenge for this technology is cloning extensive identical repeat sequences (Gaj et al. 2013). Several methods have been explored to overcome this problem, such as the 'Golden Gate' assembly method (Cermak et al. 2011), high-throughput solid-phase assembly (Briggs et al. 2012) and ligation-independent cloning techniques (Schmid-Burgk et al. 2013).

Clustered regularly interspaced palindromic repeat/associated systems

The best-known gene editing method in the field of artificial nuclease-based gene manipulation is CRISPR/Cas9.

Mechanism of CRISPR/Cas systems

CRISPR/Cas systems in bacteria and archaebacteria play a biological role in adaptive immunity systems against foreign DNA (Ran et al. 2013a; Doudna and Charpentier 2014). After a virus or phage injects DNA into the bacterium, parts of its DNA can be integrated into the CRISPR array in the genome (Wang et al. 2014). When the foreign DNA invades the

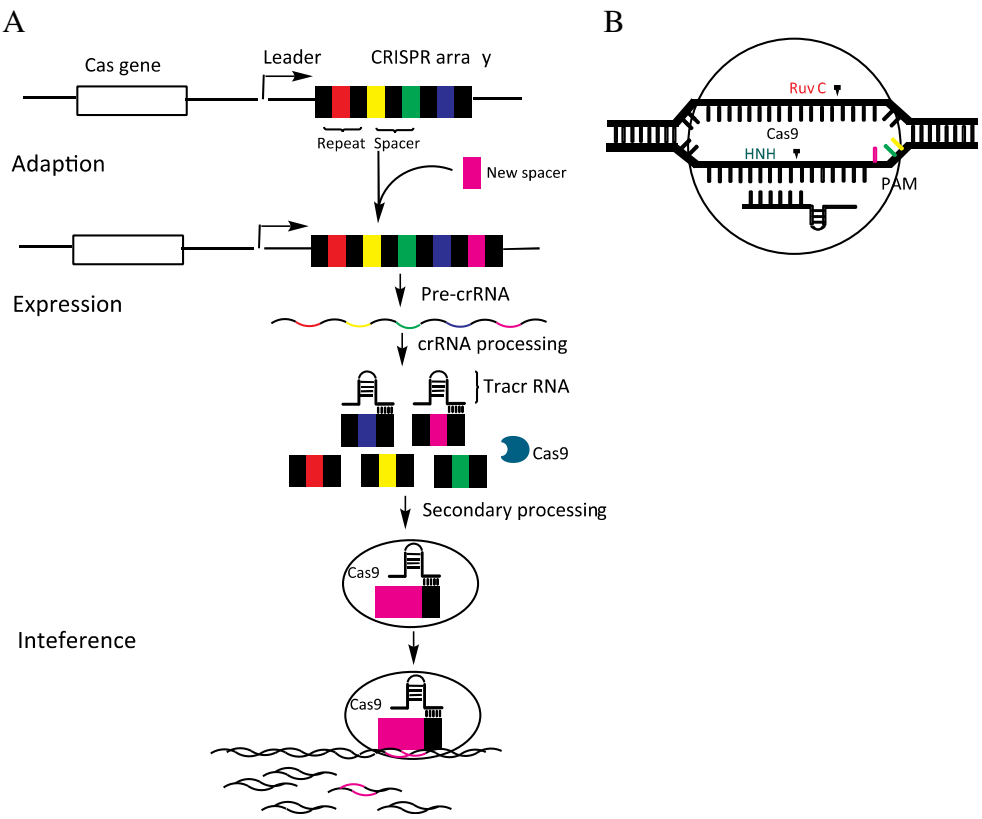

Fig. 5 Schematic overview of a CRISPR/Cas system. **a** Acquisition of the type II CRISPR/Cas system. The invaded DNA is processed as new spacer into the CRISPR array after the first attack. When the same DNA starts a second attack, the spacer expresses into the crRNA-tracr RNA complex and leads the Cas9 protein to the invading DNA and cleavage on the corresponding sequence. **b** Mechanisms of the type II CRISPR/Cas system. There is only Cas9 in a type II CRISPR/Cas system. Cas9 has two function domains: HNH, cleaving the responding sequence attached with sgRNA, and RuvC, cleaving the remaining complementary sequence. The PAM sites are usually upstream of the cleavage sites

bacterium again, the mature CRISPR RNA (crRNA) combined with a trans-activating RAN (tracrRNA) can combine and lead the Cas9 protein to degrade the DNA based on complementary sequencing (Ran et al. 2013b; Hsu et al. 2014). The process is summarised in Fig. 5.

Of the three CRISPR/Cas systems, type II CRISPR/Cas has been extensively explored for its simple mono-protein composition (Bassett et al. 2013; Friedland et al. 2013; Gratz et al. 2013). After a linker loop is added between the crRNA and tracrRNA, more convenient single-strand RNA is generated for gene editing in various cells and organisms (Bassett et al. 2013; Jinek et al. 2012). The first specific 20-nucleotide at the 5′ end of the gRNA corresponding to the tracrRNA decides the target position, and the remaining sequence at the 3′ end forms a stem-loop structure that is necessary for the activity of Cas9 protein. Due to the low content of sgRNA, a plasmid can be designed that will encode multiple sgRNAs. Multigene manipulation can thus be easily achieved, which makes unnecessary the complex procedure used in the above two nucleases guided by proteins (Shalem et al. 2014).

The commonly used CRISPR/Cas9 systems have two characteristics: they are protospacer adjacent motif (PAM) dependent and act up to six base pairs off-target. The mechanism can be understood in terms of its biological role in defending against foreign DNA. Kleinstiver et al. modified PAM sites in human cells and broadened the targeting range of *Staphylococcus aureus* Cas9, although off-target effect remains the same (Kleinstiver et al. 2015).

Many studies have been performed to improve Cas9 specificity and reduce off-target effects, including reducing the active Cas9 amount (Davis et al. 2015; Hsu et al. 2013), using inactivated Cas9 such as Cas9 nickase combined with *Fok*I nuclease domain (Guilinger et al. 2014; Tsai et al. 2014) and using a truncated guide sequence (Kleinstiver et al. 2015), among others (Slaymaker et al. 2016).

Application of CRISPR/Cas systems

Many biotechnological companies, such as Addgene, offer an online service for PAM sequence searching and sgRNA construction and provide synthesised products. Researchers have already constructed specific plasmids used to establish the CRISPR/Cas9 system in filamentous fungi. The four plasmids AFUM_pyrG, AN_argB, bleR and hygR each carry a common selection marker to allow convenient operation in different filamentous fungi (Nødvig et al. 2015).

CRISPR/Cas9 systems have been established in several filamentous fungi since 2015, and the list continues to grow. The first was *Trichoderma reesei*. A controllable and conditional CRISPR/Cas9 system was established and used to disrupt the *ura5* gene, and a 200-bp homologous length can induce site-specific mutations with 93% recombination

Table 4 Filamentous fungi with CRISPR/cas9 systems established

Species		Year	Gene editing description	Reference
Trichoderma				
	T. reesei	2015	Gene disruption and gene overexpression	(Liu et al. 2015)
Aspergillus				
	A. aculeatus	2015	Gene mutation	(Nødvig et al. 2015)
	A. brasiliensis	2015	Gene mutation	(Nødvig et al. 2015)
	A. carbonarius	2015	Gene mutation	(Nødvig et al. 2015)
	A. luchuensis	2015	Gene mutation	(Nødvig et al. 2015)
	A. niger	2015	Gene mutation	(Nødvig et al. 2015)
	A. nidulans	2015	Gene mutation	(Nødvig et al. 2015)
	A. tubingensis	2015	Gene mutation	(Nødvig et al. 2015)
	A. oryzae	2015	Gene mutation	(Katayama et al. 2016)
Penicillium				
	P. chrysogenum	2016	Gene replacement and gene knock-in	(Pohl et al. 2016)
Neurospora	*N. crassa*	2015	Gene replacement	(Matsu-ura et al. 2015)
Phytophthora	*P. sojae*	2016	Gene disruption and gene replacement	(Fang and Tyler 2016)
Pyricularia	*P. oryzae*	2015	Gene replacement	(Arazoe et al. 2015)
Ustilago	*U. maydis*	2016	Gene disruption	(Schuster et al. 2016)

frequency with the help of the CRISPR/Cas9 system (Liu et al. 2015). This system has been established in *Trichoderma*, *Aspergillus*, *Penicillium* and the model organism *Neurospora crassa* (Table 4).

Other applications of the CRISPR/Cas9 systems used in bacteria, such as CRISPR interference (Cleto et al. 2016; Qi et al. 2013) and activation (Cheng et al. 2013), may also be used for filamentous fungi because the inactive form of Cas9 nucleases also exhibits versatile abilities. With inactivation of either of the functional parts of the HNH and RuvC domain, the Cas9 nucleases become nickases that induce single-strand breaks. With both inactive domains, Cas9 nucleases become repressor-like proteins that can block the transcription progress, which is called CRISPR interference (CRISPRi) (Larson et al. 2013). If a regulatory part, a repressor or an enhancer, is combined with a Cas9 protein with DNA targeting ability, a single gene or a series of genes can be regulated. Researchers have compared the effect of CRISPRi and shRNA in the identification of essential genes in RT-112 cells and found that a CRISPR-based screening method performed the best (Evers et al. 2016).

Integration of Cas9 protein and sgRNA

The key challenge to the establishment of this system in filamentous fungi is the method of integrating the Cas9 protein and sgRNA into the host. Methods include DNA and mRNA techniques. Both components can be co-transformed by one plasmid or solo transformed by being transformed twice. In most filamentous fungi, the Cas9 DNA is integrated into the host's genome, and some filamentous fungi such as *Ustilago maydis* use an auto-replicating plasmid with Cas9 DNA in the cytoplasm. The auto-replicating plasmid allows transient expression of Cas9 to avoid the disadvantage of permanent Cas9 (Schuster et al. 2016), which was found to aggravate the off-target effect.

In addition, a RNA-guided endonuclease technique using a preassembled duplex combined with purified Cas9 proteins has been successfully used in the filamentous fungus *P. chrysogenum* (Pohl et al. 2016) and in human cells, zebrafish embryos, bacteria (Kim et al. 2014) and plants (Woo et al. 2015). This preassembled ribonucleoproteins method is able to improve on-target efficiency and reduce off-target inefficiency compared with the traditional plasmid transformation method, with which it is difficult to successfully integrate the Cas9 protein into the genome and in which continuous expression of the Cas9 protein can aggravate the off-target effects.

SgRNA

RNA polymerase III promoters, such as U6 and T7 promoters, drive the expression of sgRNA. The endogenous RNA polymerase III promoter may not exist in filamentous fungi, so a heterogenous RNA polymerase III promoter, usually U6, is often used. Alternative methods, such as using sgRNA derived from the RNA polymerase II promoter, and RNA

processing strategies have also shown the capability of sgRNA synthesis.

The sgRNA derived from the RNA polymerase II promoter in *P. oryzae* has the ability to guide the Cas9 protein but shows less activity than sgRNA derived from the RNA polymerase III promoter (Arazoe et al. 2015). Researchers have also used ribozymes to design the artificial gene RGR (ribozyme-RNA-ribozyme), whose transcripts can undergo self-catalysed cleavage and release the designed gRNA using the RNA polymerase II promoter in vitro and in yeast. The results showed efficient Cas9-mediated target DNA cleavage (Gao and Zhao 2014). Other integrated RNA methods, including RNA-triple-helix structures, introns and microRNAs, have also been attempted in human cells (Nissim et al. 2014), and those methods may also be capable of operating well in filamentous fungi.

RNA technology

Besides the molecular tools used for DNA manipulation, some RNA technology which can verify gene function through disturbing mRNA translation was also applied in filamentous fungi. Those technologies include RNA interference and ribozymes.

RNA interference

RNA interference (RNAi) is an available method for the verification of gene function when the necessary gene knock methods are lacking. In filamentous fungi, RNA quelling was first discovered in *N. crassa*; six pathways produced various small RNAs (Li et al. 2010). The mechanisms of two RNAi-related phenomena including quelling and meiotic silencing of *N. crassa* were demonstrated by Romano et al. (Romano and Macino 1992). In summary, the aberrant RNA can trigger cells to inactivate the connate mRNA, thus quelling gene expression in the cell.

The RNAi technology has been successfully used in more than 30 species of filamentous fungi and fungus-like organisms such as *Mucor* and *Aspergillus*. Kuck et al. gave the list of filamentous fungi with successful application of the RANi technology in 2010 (Kück and Hoff 2010). Some filamentous fungi, such as *Mortierella alpina* (Chen et al. 2015), are added into the list in the following years.

The results of RNA interference cannot be predicted and varies among experiments and laboratories. This method is not applicable to some filamentous fungi, such as *U. maydis*, because the necessary component in the RNAi silencing pathway is absent (Jiang et al. 2013).

Ribozymes

Ribozymes are special RNAs capable of catalysing RNA. Among the different kinds of natural ribozymes, hammerhead ribozymes and hairpin ribozymes are extensively used due to their small size and high cleavage activity. Up to now, only the hammerhead ribozymes were successfully used in filamentous fungi. The first reported case was in *Aspergillus giganteus* with seven different hammerhead ribozymes designed targeting the mRNA of the beta-glucuronidase transcript (uidA). (Mueller et al. 2006) The result showed that the ribozymes could reduce uidA expression up to 100%.

Although various forms of products for small RNAs are available, such as short-hairpin RNA (shRNA) (Hannon 2002), double-strand RNA transcribed in both directions under dual promoters, antisense single-strand RNA (Hamilton and Baulcombe 1999) and directly synthetic double-strand RNA, different kinds of filamentous fungi have different levels of ability to take up foreign RNAs, thus leading to different silencing effects. Moreover, the heritability of this molecular tool is not stable. Those disadvantages need to be taken into consideration before choosing the right gene manipulation tools.

Summary and outlook

All gene manipulation systems require choices about DNA vectors, transformation methods and gene editing strategies. Gene manipulation plays a key role in this gene function exploration generation. The gene manipulation methods for prokaryotes have developed more quickly than those for filamentous fungi. Because of the complexities of ploidy, propagation and the mycelial structure of filamentous fungi, the search for a versatile, effective and stable genetic tool for use in filamentous fungi faces many challenges. The special composition of fungal cell wall makes it difficult for recombinant DNA to enter the host. These factors may account for the slow progress in the development of molecular genetic tools for use in filamentous fungi. The further development of these molecular tools still faces these difficulties.

As for gene editing strategies, the commonly used homologous recombination method and RNA interference are mainly used to uncover the role of a single gene among multiple pathway genes due to the technological barriers. With the development of gene editing technologies, new tools such as TALENs and the CRISPR/Cas system, which simplify the gene manipulation process and improve gene targeting efficiencies, have arisen to meet the needs of a new generation. Gene manipulation with the CRISPR/Cas9 system in filamentous fungi mainly involves gene mutation, but other types of gene manipulation such as gene motivation and gene interference are likely to be applied in the future. The RNA editing

tool C2c2, which is already used in bacteria (Abudayyeh et al. 2016) and was derived from the CRISPR/Cas system, may also be useful in filamentous fungi. The novel CRISPR/Cas systems CRISPR/CasX and CRISPR/CasY (Burstein et al. 2016) offer new choices for the future, and gene editing tools that use DNA-guided artificial nucleases (Qi et al. 2016) may open up new techniques for the gene manipulation field.

Through these gene editing strategies, further eukaryotic regulatory mechanisms of anabolism, catabolism, growth and phenotype can be elucidated and many more filamentous species may become available as 'cell factories' for use in the medical, agricultural and food industries.

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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 animals performed by any of the authors.

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