Applications of CRISPR/Cas Gene-Editing Technology in Fungi

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Abstract

Genome editing technology develop fast in recent years. The traditional gene-editing methods, including homologous recombination, zinc finger endonuclease, and transcription activator-like effector nuclease and so on, which have greatly promoted the research of genetics and molecular biology, have gradually showed their limitations such as low efficiency, high error rate, and complex design. In 2012,a new gene-editing technology, the CRISPR/Cas9 system, was setup based on the research of the immune responses to viruses from archaea and bacteria. Due to its advantages of high target efficiency, simple primer design, and wide application, CRISPR/Cas9 system, whose developers are awared the Nobel Prize in Chemistry this year, has become the dominant genomic editing technology in global academia and some pharmaceuticals. Here we briefly introduce the CRISPR/Cas system and its main applications in yeast, filamentous fungi and macrofungi, including single nucleotide, polygene and polyploid editing, yeast chromosome construction, yeast genome and yeast library construction, CRISPRa/CRISPRi-mediated, CRISPR platform of non-traditional yeast and regulation of metabolic pathway, to highlight the possible applications on fungal infection treatment and to promote the transformation and application of the CRISPR/Cas system in fungi.

1. Introduction

The CRISPR/Cas system was developed from investigations of archaeal and bacterial immune systems (Horvath and Barrangou 2010). The invasion of foreign genetic materials, such as phage DNA or plasmids, induces an autoimmune reaction, including the formation of a complex able to cut this material and thereby exert a protective effect (van der Oost et al. 2014). Research on CRISPR can be traced back to 1987. Ishino et al. (1987) found abnormal repeats in a noncoding region after a termination codon in *Escherichia coli* K12, in the first report of interval tandem repeats. The sequence was named clustered regularly interspaced short palindromic repeats (CRISPR) (Jansen et al. 2002). In 2012, Jinek et al. (2012) modified the CRISPR/Cas9 system of *Streptococcus pyogenes* to enable gene modification and thus the CRISPR/Cas system was introduced as a new gene-editing technology and an alternative to homologous recombination, zinc finger endonucleases (ZFNs), and transcription activator-like effector nucleases (TALENs). In 2013, Zhang Feng first achieved genome editing by the CRISPR/Cas9 system in mammalian and human cells (Cong et al. 2013). Since then, the CRISPR/Cas9 system has been rapidly applied to bacteria (Westbrook et al. 2016), fungi (DiCarlo et al. 2013), animals (Hruscha et al. 2013; Li et al. 2013; Fan et al. 2014; Chen et al. 2015; Wan et al. 2015; Zou et al. 2015), human cells (Mali et al. 2013), plants (Ma et al. 2015; Zhu et al. 2020; Pan et al. 2021), and other organisms owing to its low cost, simple structure, accuracy, and high efficiency. The modification types include knock-ins, deletions, multiple target site mutations, and large fragment deletions (Mohanraju et al. 2016). The CRISPR/Cas9 system has become a well characterized gene-editing technology and is still being improved and optimized to improve the activity and specificity of editing and to expand the scope of application.

CRISPR/Cas systems can be divided into two types according to the Cas effector proteins (Makarova et al. 2015). In the first system, 4–7 Cas proteins form a multisubunit crRNA-protein complex, such as the

CRISPR-associated complex for antiviral defense (cascade) (Mohanraju et al. 2016). In the second system, a single effector protein is used for target cleavage, such as Cas9 or Cpfl (Makarova et al. 2015). Each system includes three types—type I, III, and IV in the class 1 system and type II, V, and VI in the class 2 system (Ishino et al. 2018; Anzalone et al. 2020). The class 1 system is the most common type in archaea and bacteria (Makarova et al. 2015). However, due to the complexity and diversity of the cascade, its application in gene-editing technology is limited to some extent (Anzalone et al. 2020). The class 2 system accounts for about 10% of discovered systems and is found almost exclusively in bacteria. It can be divided into 10 subtypes (Koonin et al. 2017), including cas9 in type II, cpf1 (now widely known as cas12a) in type V, and some predicted V-U subtypes (Shmakov et al. 2017). At present, type II is the most widely used CRISPR/Cas9 system. It is composed of a crRNA, tracrRNA, and Cas9 protein. It cleaves the target gene by recognizing the site with a protospacer adjacent motif (PAM) sequence 5'-NGG-3' (Anders et al. 2014). The CRISPR/Cas9 system is not only used for gene knockouts; mutations in the D10A/H840A site of the cas9 protein yield dead Cas9 (dCas9), which loses its shearing activity and retains the ability to recognize target genes (Deltcheva et al. 2011). It have been used for cargo delivery and the modification of gene expression (Li et al. 2019; Nguyen et al. 2021; Shin et al. 2021).

CRISPR/Cas system in fungi is usually carried out by modifying the Class 2 system (Cas9/Cas12a) of CRISPR from bacteria (Table 1). This review discusses the application of the CRISPR/Cas system in yeast, filamentous fungi, and macrofungi, including polygene and polyploid editing, the synthesis of yeast chromosomes, genome-scale engineering and library creation, CRISPR activation/interaction, CRISPR-mediated non-traditional yeast editing, regulation of metabolic pathways, and the treatment of fungal infections (Fig. 1).

2. Strategies For The Development Of Crispr/cas9 Tools In Various Fungi

2.1 Yeast

Yeast, monocellular eukaryotes, are easy to culture and grow rapidly. Compared with other eukaryotes, yeast have a simpler genetic background. Yeast are also used in industrial fermentation owing to their easy operation and safety. CRISPR technology has been widely used in yeast for the following main objectives: 1) yeast polygene and polyploid knockout, 2) the synthesis of yeast chromosomes, 3) genome-scale engineering and library creation, 4) gene integration, heterologous expression, and metabolic pathway regulation, 5) CRISPR-mediated activation/interactions, 6) non-traditional gene editing and explorations of virulence mechanisms.

2.1.1 Polygene and polyploid editing of yeast

Rapid advances in gene sequencing technology have resulted in substantial improvements in our understanding of microbial genomes. The existence and distribution of functional genes can be

deciphered by the gene-editing method, providing a basis for in-depth studies of fungal microbiome functions (Aguilar-Pontes et al. 2014). Traditional yeast polygenic editing requires multiple rounds of selective pressure (Adames et al. 2019). CRISPR technology can reduce or even avoid the use of resistance. In 2013, DiCarlo et al. (2013) first introduced the CRISPR/Cas9 system into *Saccharomyces cerevisiae*, demonstrating an improvement in the efficiency of double-strand break (DSB) repair by gene recombination by 130-fold. Bao et al. (2015) developed a homology-integrated CRISPR/Cas (HI-CRISPR) system in *S. cerevisiae*. They realized one-step multigene disruption by concatenating different guide RNAs (gRNAs) on a vector, with an efficiency of 27–87%. Mans et al. (2015) transformed three plasmids by the *in vitro* assembly of plasmids containing two gRNAs, and simultaneously produced six gene deletion strains of *S. cerevisiae*. The CRISPR/Cas system has also been used for the multiple genome engineering of polyploid industrial yeasts. Zhang et al. (2014) used Cas9 to knock out four genes of *S. cerevisiae* ATCC 4124 with 15–60% efficiency, and four nutrient-deficient strains were obtained. Lian et al. (2017) expressed gRNA by high-copy plasmids and then transformed the plasmids into a diploid strain (Ethanol Red) and a triploid line (ATCC 4124). Four genes were knocked out in a single step with an efficiency of 100%. Additionally, Li et al. (2018) found that Cpf1 from *Francisella novivida* (FnCpf1) can target DNA fragments assembled *in vivo* for singleplex, doubleplex, and tripleplex genomic integration, with efficiencies of 95%, 52%, and 43%, respectively. Yang et al. (2020) used CRISPR/Cpf1 to target the *CAN1* and *URA3* genes of *Yarrowia lipolytica*, obtaining efficiencies of up to 93% and 96%. Zhao et al. (2020) also applied the CRISPR/Cas12a system to *Schizosaccharomyces pombe*. Because Cpf1 does not need tracrRNA and lacks the HNH endonuclease domain present in Cas9, it is well-suited to yeast multiplex gene editing (Zetsche et al. 2015; Swiat et al. 2017; Zetsche et al. 2017; Li et al. 2018). Multi-gene editing technology based on CRISPR can be used to create complex traits and explore gene regulatory networks.

2.1.2 Synthesis of yeast chromosomes

Variation in chromosome structure plays an important role in the diversity of biological characters. The synthetic yeast genome, designated Sc2.0, was an international cooperative project aimed at designing and constructing the first fully chemically synthesized eukaryotic cell with 16 chromosomes (Richardson et al. 2017). CRISPR/Cas9 provides a method for the redesign and construction of *S. cerevisiae* chromosomes. Chromosome drives via CRISPR/Cas9 enable the biased inheritance of complex genetic traits on a chromosomal scale in yeast (Xu et al. 2020). Luo et al. (2018) successfully fused yeast chromosomes using CRISPR/Cas9 and produced a series of homologous strains with 2 to 16 chromosomes. Xie et al. (2017) constructed a ring synthetic chromosome □ of *S. cerevisiae* by CRISPR technology. Shao et al. (2018) used CRISPR/Cas9 to efficiently knock out redundant centromeres and telomeres of chromosomes for fusion in *S. cerevisiae* and used homologous recombination to realize 15 rounds of chromosome fusion. Finally, 16 natural chromosomes of a haploid *S. cerevisiae* were artificially fused into the yeast strain SY14 with a single chromosome. In 2019, the CRISPR/Cas9 method was further used to induce DSBs in the proximal regions of two telomeres of the SY14 linear chromosome, and the ends of two DSBs were connected with donor DNA fragments by endogenous homologous recombination to generate a new strain with a single circular chromosome, designated SY15 (Shao et al.

2019). The rearrangement of yeast chromosomes clearly establishes the effectiveness of CRISPR/Cas-based systems for evaluating the structure and function of genes in eukaryotic evolution.

2.1.3 Genome-scale engineering of yeast and library creation

The programmable control of gene expression is essential for understanding gene function, regulating cell behavior, and developing therapeutic methods. Genetic screening can be used to study the functions of multiple genes simultaneously in a high-throughput manner. CRISPR/Cas9 has been used as a screening strategy to induce mutations and assess gene function. In 2018, Bao et al. (2018) developed CRISPR/Cas9 and homology-directed-repair assisted genome-scale engineering (CHAnGE), which can be used to edit the whole genome of *S. cerevisiae* at the single nucleotide level. This method can rapidly generate thousands of specific mutant yeasts. CHAnGE can generate single base pair changes on the whole chromosome and minimize the impact on the function of adjacent genes with unprecedented accuracy. The authors have also established a gene knockout yeast library using the system to improve the production of heterologous natural products. Li et al. (2019) created the CRISPR Activation Library based on the gene regulation system of dCas9. They identified a high-temperature resistant yeast with a new gene regulatory mechanism by the construction of a library with 260 sgRNAs. Guo et al. (2018) used CRISPR/Cas9 to induce DSBs in yeast target genes and provided a donor template with programmed mutations for homology-directed repair (HDR), realizing the high-throughput creation and functional analysis of a yeast DNA sequence variant library. Ferreira et al. (2019) used dCas9-VPR and a library of 3194 gRNAs targeting 168 genes to screen targets for enhancing the flux toward cytosolic malonyl-CoA, significantly increasing the production of 3-hydroxypropionic acid (3-HP). Buchmuller et al. (2019) constructed a yeast library by CRISPR/Cpf1-assisted tag library engineering (CASTLING). The yeast library is an ideal model for studies in systematic biology, and the CRISPR/Cas system is a practical tool for the establishment of systematic libraries and for genome-scale research.

2.1.4 Gene integration, heterologous expression, and metabolic pathway regulation

Balancing gene expression levels in endogenous or exogenous metabolic pathways is an important way to improve the yield and efficiency of target products in yeast (Idiris et al. 2010). Eukaryotes can produce abundant secondary metabolites (Jiang et al. 2021; Liu et al. 2021; Sharma et al. 2021). There secondary metabolic clusters are typically silent and transcriptionally inactive under laboratory conditions(Osbourn 2010; Brakhage and Schroeckh 2011; Keller 2019). These silent gene clusters have been cloned and transferred into *S. cerevisiae* or other microorganisms to activate the expression of gene clusters (Keller 2019). The target gene was cleaved by a nuclease to form DSBs and then repaired by HDR or non-homologous end joining (NHEJ) (Ceccaldi et al. 2016). NHEJ is feasible for the construction of mutant libraries, since repair is random. However, for rational metabolic engineering, HR is preferred owing to its precision and predictability. CRISPR/Cas enables precise gene editing, which can undoubtedly accelerate the heterologous expression of silent genes (Jakounas et al. 2015; Xu et al. 2016). Both HR and NHEJ activity via CRISPR/Cas9-induced oligodeoxynucleotide (ODN)-mediated DSB repair have been quantitatively measured (Du J et al. 2018). Horwitz et al. (2015) used the CRISPR system to integrate six

DNA fragments carrying 11 genes with a total length of 24 kb in *S. cerevisiae* and finally established prototype pathways for muconic acid production. In 2016, Jessop-Fabre et al. (2016) constructed the EasyClone-MarkerFree plasmid toolbox, which can simultaneously insert 1–3 DNA fragments into the genome of *S. cerevisiae* without using selective markers. Ronda et al. (2015) combined the stability and versatility of the EasyClone vector system with the precision and efficiency of CRISPR/Cas9 to efficiently integrate three genes involved in the β-carotene pathway at three different sites on three chromosomes in *S. cerevisiae*, thus improving the production of β-carotene. Shi et al. (2015) constructed a Delta integration CRISPR/Cas (Di-CRISPR) platform for multi-copy gene integration, which can efficiently integrate large-scale biochemical pathways without labeling. Utilizing this method, they achieved the unprecedented one-step integration of 18-copy genomes of 24 kb and successfully constructed a yeast strain capable of directly using xylose to produce (*R*,*R*)-2,3-butanediol (BDO). Wan et al. (2018) overexpressed *MRP8,* which encodes a mitochondrial ribosomal protein, in recombinant *S. cerevisiae* Y294 by CRISPR/Cas9 technology, thereby increasing extracellular Cbh1 enzyme activity by 80%. Sun et al. (2020) successfully knocked out two copies of the gene encoding isocitrate dehydrogenase (ICD) in *Pichia kudriavzevii* and integrated *At_CAD* and *Pk_MTTA* in the mutant strain to increase the yield of itaconic acid (IA). CRISPR/Cas9 technology has also been used to remove genes with adverse effects. For example, Chin et al. (2016) mutated the yeast *CAR1* gene, encoding arginase, by CRISPR/Cas9 technology, thus reducing carcinogenic ethyl carbamate (EC) produced during ethanol fermentation.

Due to differences in the recognition of promoters among eukaryotic taxa, silent gene clusters cannot be activated when they are directly cloned and transferred into heterologous hosts. The CRISPR/Cas9 system can be used to change the original regulatory elements to achieve the heterologous expression of these genes. Kang et al. (2016) described an improved yeast-based promoter engineering platform (mCRISTAR) that combines CRISPR/Cas9 and the transformation-associated recombination (TAR) (Yamanaka et al. 2014) to replace natural promoters with combinatorial promoters. CRISPR/Cas9 mediates DSBs to form linear DNA fragments at the target promoter and then integrates the homologous arm with biosynthetic gene clusters (BGCs) by homologous recombination in yeast cells. Up to 32 promoters can be inserted into a single natural BGC by four rounds of mCRISTAR using four auxotrophic markers commonly used in yeast, and silent gene clusters can be transcriptionally activated (Kang et al. 2016).

2.1.5 CRISPR activation (CRISPRa)/interaction (CRISPRi) in yeast

Recently, D10A and H804A mutations were introduced into the Ruvc and HNH domains of the Cas9 protein in the CRISPR system to obtain nuclease-deficient Cas9 (dCas9). By fusing dCas9 with different types of transcriptional regulatory domains, such as transcriptional inhibitors, activators, or epigenetic modification enzymes, dCas9 proteins can target specific sites of target genes, resulting in different modes of gene regulation (Larson et al. 2013; Qi et al. 2013) (Fig. 2). Changes in gene expression and activity can be obtained by CRISPRa/CRISPRi in yeast. dCas9-Mxi1 and dCas9-VPR are the most commonly used regulators (Jensen 2018). Gilbert et al. (2013) fused dCas9 with Mxi1, a mammalian transcription inhibition domain, and targeted the *Tef1* promoter. They found that the inhibitory effect of

dCas9 on reporter gene expression increased from 18-fold for dCas9 alone to 53-fold, indicating that dCas9 binding to transcriptional regulators is highly effective. Schwartz et al. (2017) applied the CRISPRi system to *Yarrowia lipolytica*, designed sgRNAs for the TSS and TATA box of target gene promoter regions, and successfully inhibited 8 of 9 target genes. They also found that dCas9-mxi1 increased the inhibition of *KU80* gene transcription from 38% for dCas9 to 87%. Vanegas et al. (2017) combined Cas9 and dCas9 into a SWITCH dynamic CRISPR tool where switching mechanism is based on the recombination of dCas9 after Cas9 is directed to cleave its own gene sequence. The tool enables *S. cerevisiae* strains to alternate between genetic engineering and metabolic pathway control states, enabling the accurate control of multiple genes of *S. cerevisiae*.

Farzadfard et al. (2013) fused dCas9 with VP64 (a commonly used eukaryotic transcription activator domain) in *S. cerevisiae* and targeted to a minimal *CYC1* promoter (pCYC1m). The expression of gRNAs in different regions of pCYC1m resulted in different levels of fluorescent *GFP* reporter gene activation and inhibition, indicating that dCas9/dCas9-VP64 can interfere with the formation of the transcription initiation complex by targeting different positions in endogenous promoters. Lian et al. (2017) tested a combination of dCas proteins from different sources (*S. pyogenes*, *Staphylococcus aureus*, *Streptococcus thermophiles*, and *Lachnospiraceae bacterium*) , and multiple transcriptional activation or inhibitory domains and finally constructed a three-phase gene regulatory strategy for *S. cerevisiae* based on dLbCpf1-VP (CRISPRa), dSpCas9-RD1152 (CRISPRi), and SaCas9 (CRISPRd), known as CRISPR-AID. Using this system, they transformed a single plasmid into yeast to simultaneously induce a 5-fold increase in red fluorescent protein, 5-fold inhibition of yellow fluorescent protein, and 95% deletion of the endogenous gene. These studies demonstrate that CRISPR/Cas is a powerful tool for fungal genetic screening. CRISPRa/i reversibly regulates the expression of target genes rather than mutation, which reduces errors in gene repair.

2.1.6 Development of a CRISPR gene editing platform in non-traditional yeast and exploration of virulence mechanisms

The successful application of CRISPR/Cas9 in *S. cerevisiae* genome editing has prompted interest in the genetic manipulation of various non-traditional yeasts (Arras et al. 2016; Schwartz et al. 2016; Raschmanová et al. 2018). In particular, the CRISPR/Cas9 system has been developed to study the virulence mechanisms of *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Cryptococcus neoformans,* and other clinically related pathogenic yeasts (Vyas et al. 2015; Enkler et al. 2016; Lombardi et al. 2017; Wang 2018). In 2015, Vyas et al. (2015) first applied the CRISPR/Cas9 system to gene editing in *C. albicans*. By introducing the Cas9 protein and sgRNA, the target gene was driven by RNA polymerase III promoter SNR52, and a repair template containing a stop codon was provided, resulting in the production of homozygous mutants in one transformation. Xu et al. (2018) successfully obtained a homozygous inactivation mutant of *CaMIT1* by the CRISPR/Cas9 method. The mutant strain was sensitive to calcium and lithium ions, sodium dodecyl sulfate, clotrimazole, and ketoconazole but tolerant of Congo red. Min et al. (2016) showed that CRISPR/Cas9 gene elements can play a transient role in *C. albicans* without stable integration into the genome, addressing the concern that Cas9 may cause long-

term adverse effects (such as off-target effects) in the *C. albicans* genome. Huang et al. (2017) developed a marker recovery strategy using CRISPR/Cas9. Two marker genes can be used to sequentially screen homozygous deletion mutants with three or more genes in the same strain. Nguyen et al. (2017) removed CRISPR and nourseothricin (NAT) markers from the genome by the *SAT1* flipper system after determining the target site modification of *C. albicans*, thus allowing the next round of unlabeled genome editing. Grahl et al. (2017) used a purified Cas9 protein, crRNA, and tracrRNA to form ribonucleoproteins (RNPs) to modify the genome of three non-*C. albicans Candida* (NCAC), resolving the low promoter activity of the CRISPR/Cas9 system for different species-specific patterns of gene expression. Rybak et al. (2020) introduced *TAC1B* mutations from drug-resistant clinical isolates into the fluconazole-susceptible *C. auris* strains by an RNP-mediated transformation system and found that *TAC1B* mutations explain the observed increase in fluconazole resistance. Lombardi et al. (2017) described the first CRISPR/Cas9 editing system based on plasmids with an autonomous replication sequence (ARS) in *C. parapsilosis*. The gRNA is released between two ribozymes (Hammerhead and hepatitis delta virus [HDV]) for multiple editing of the target gene. In 2019, they improved the system so that the gRNA could be introduced in a single cloning step and released by cleavage between a tRNA and a ribozyme (Lombardi et al. 2019). This method was used for efficient gene editing in *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*. Zhang et al. (2019) developed a CRISPR/Cas9 system for genome editing in *C. tropicalis*, demonstrating the efficient deletion of single or double genes in 9 days, 17 fewer days than required for the traditional *SAT1* flipper strategy. This method can be used to promote the assembly and stable integration of multiple DNA fragments into a target site in *C. tropicalis*. Zoppo et al. (2020) used CRISPR/Cas9 technology to study the virulence of Als in *C. parapsilosis*. Ibrahim et al. (2020) constructed an episomal vector for the expression of Cas9 and sgRNA by using an ARS isolated from *C. aaseri* SH14 and used a single sgRNA with 70% efficiency to destroy six copies of acyl-CoA oxidase genes (*AOX2*, *AOX4,* and *AOX5*) in diploid cells simultaneously.

2.2 Filamentous fungi

Filamentous fungi play important roles in the biomass cycle of ecosystems. They can produce a variety of secondary metabolites, such as cellulase, pectinase, and protease (Chandel et al. 2012; Li et al. 2020). These fungi can result in the deterioration of buildings, food, and feed and can even cause fatal diseases in humans (Nevalainen et al. 2015; Köhler et al. 2017; Avery et al. 2019). Before the CRISPR/Cas system was proposed, gene editing of these fungi was a big issue in biology (Wang et al. 2017). The CRISPR/Cas9 system has provided a convenient tool for studies of filamentous fungi, such as analyses of gene function, pathogenic mechanisms, and metabolic pathways and the development of methods to increase product yields.

2.2.1 Unlocking gene function

CRISPR/Cas9 technology has been used for genome editing in various filamentous fungi (Nødvig et al. 2015; Wang and Coleman 2019), such as *Trichoderma reesei* (Liu et al. 2015), *Neurospora crassa* (Matsu-Ura T 2015), *Aspergillus oryzae* (Katayama et al. 2015), *Aspergillus niger* (Kuivanen et al. 2016),

Magnaporthe oryzae (Foster et al. 2018), *Myceliophthora thermophila* (Liu et al. 2017), *Aspergillus nidulans* (Zhang et al. 2016), *Ustilago maydis* (Schuster et al. 2016), *Mucor circulalloides* (Nagy et al. 2017), *Phytophthora sojae* (Miao et al. 2020), *Aspergillus fumigatus* (Fuller et al. 2015), and *Penicillium chrysogenum* (Pohl et al. 2016). The Cas9-gRNA complex assembled *in vitro* or Cas9 and gRNA expressed *in vivo* have been employed to deactivate genes, followed by comparative analyses of metabolites, cell morphology, toxicity, and other properties between the wild-type and deletion strains to verify gene functions .

Liu et al. (2015) edited *T. reesei* genes by optimizing the transcription of the Cas9 protein and *in vitro* gRNA transcription and successfully applied the CRISPR/Cas9 system to filamentous fungi for the first time in 2015. Nielsen et al. (2017) discovered a new gene in *Talaromyces atroroseus* responsible for the production of polyketide-nonribosomal peptide hybrid products using CRISPR/Cas9 technology. Subsequently, the application of the CRISPR/Cas9 system in fungi has been continuously optimized. Zhang et al. (2016) used the CRISPR/Cas9 system to accurately integrate the *GFP* gene into predicted sites in clinical isolates of *A. fumigatus* without inserting markers by microhomology-mediated end joining (MMEJ), and the integration efficiency was 95–100% using only a 35 bp homologous arm.

Nødvig et al. (2015) constructed a general vector and used *Tef1* and *GpdA* promoters to drive the expression of Cas9 and gRNA, respectively. By adding nuclease sequences HH and HDV at both ends of the gRNA, gene knockout was finally achieved in six strains of fungi, including *A. nidulans* and *A. niger*. Liu et al. (2017) used the CRISPR/Cas9 system for multiple gene editing in *M. thermophila*. The recombination efficiency of single-gene mutations was 90–95%, that of two gene mutations was 61–70%, and those of three and four gene mutations were 30% and 22%, respectively. Zheng et al. (2018) used endogenous 5S rRNA of *A. niger* to drive sgRNA transcription and used 40 bp homologous donor DNA to directly delete a 48 kb long DNA fragment from the *A. niger* genome for the first time, with a targeting efficiency of 100%.

Owing to the limited number of selective marker genes in filamentous fungi and the difficulty in multiple rounds of gene manipulation, a single screening marker recycling method in filamentous fungi using the CRISPR/Cas9 system has been established. The self-replicating cas9 plasmid in *U. maydis* and *P. chrysogenum* would be lost in the absence of resistance pressure, thus avoiding its influence on the growth of the strain (Pohl et al. 2016; Schuster et al. 2016). Furthermore, CRISPR/Cas9 has been combined with Cre/loxP to develop a marker-free fungal gene editing system. First, the CRISPR/Cas9 system is used to break a target gene, and the repair template containing the screening marker was integrated into the cleavage site; then, Cre recombinase activated by light illumination could simultaneously delete the selective marker and *CRE* (Zhang et al. 2016). Liu et al. (2019) developed a V-type CRISPR/Cas12a (AsCpf1) system in *M. thermophila*. Through three rounds of transformation with two selectable markers, nine genes involved in the cellulase production pathway were targeted. The protein productivity and lignocellulase activity of a mutant (referred to as M9) were 9.0- and 18.5-fold higher than those of the wild type. *Cas12a* was also used for gene editing in *A. nidulans* and *A. niger* (Vanegas et al. 2019).

2.2.2 Interference with metabolic pathways to obtain secondary metabolites and increase yield

Some secondary metabolites of microorganisms are important pathogenic factors contributing to human and plant diseases; however, some are also important sources of bioactive substances and drug precursor compounds (Evidente et al. 2014). Filamentous fungi have a strong metabolic capacity and can produce secondary metabolites with diverse structures, such as biocidal agents, drug precursors, and antitumor bioactive substances (Hoffmeister and Keller 2007; Osbourn 2010; Ma et al. 2016). The CRISPR/Cas system can accurately alter gene expression for functional analyses of filamentous fungal metabolic gene clusters, analyses of synthesis and regulatory mechanisms, and the activation of silent gene clusters to interfere with metabolic bypass, which is expected to improve the production and activity of secondary metabolites of filamentous fungi. Kuivanen et al. (2016) combined a transcriptomics approach and CRISPR/Cas technology to delete genes involved in galactaric acid catabolism in *A. niger* and then heterologously expressed uronate dehydrogenase, yielding a mutant able to convert pectin-rich biomass to galactaric acid in a consolidated bioprocess.

In addition to the conventional gene editing, CRISPR/Cas technology can also knock-in strong promoters or replace promoters upstream of target genes, thus activating silent gene clusters, regulating biosynthetic genes, and synthesizing corresponding metabolites. Matsu-ura et al. (2015) successfully replaced the endogenous promoter of the cellulase related gene *CLR-2* with a β-tubulin promoter in *N. crassa* using the CRISPR/Cas9 system. The mRNA expression of *CLR-2* in the mutant strain increased about 200-fold and cellulase production increased significantly. Therefore, the CRISPR/Cas system is a powerful synthetic biology tool for the control of the biosynthesis of secondary metabolites in fungi.

2.3 Macrofungi and other fungi

Macrofungi are multicellular eukaryotes; most are binucleate or multinucleated cells. The low efficiency of obtaining homozygous mutations makes it difficult to achieve the specific modification of target genes and multigene knockout or gene insertion mutations, thereby limiting basic research focused on macrofungi (Alberti et al. 2020). At present, research on CRISPR/Cas9 in macrofungi is mainly focused on the establishment of the system in different species. In 2016, Waltz et al. (2016) used CRISPR/Cas9 to knock out one of six polyphenol oxidase (PPO) genes in *Agaricus bisporus*, reducing enzyme activity by 30% and effectively slowing the rate of browning. Qin et al. (2017) first used CRISPR/Cas9 technology to disrupt the *URA3* gene of *Ganoderma lucidum*. The gene-editing efficiency of *G. lucidum* was proportional to the amount of gRNA (Qin et al. 2017). Liu et al. (2020) added an intron upstream of the *Cas9* gene, increasing the CRISPR/Cas9-mediated gene disruption frequency in *G. lucidum* by 10.6-fold. Chen et al. (2018) first applied the CRISPR/Cas9 system to *Cordyceps militaris* by using an optimized Cas9 protein and synthesized gRNA *in vitro*. In 2017, Deng et al. (2017) used CRISPR/Cas9 technology tconfirmed that a *SbaPKS* gene in *Shiraia* sp. is involved in the biosynthesis of hypocrellin, related to virulence. Sugano et al. (2017) combined the cryopreserved protoplasts and CRISPR/Cas9 technology in *Coprinopsis cinerea* to disrupt the *GFP* gene by using the *CcDed1* promoter to express Cas9. In addition, CRISPR/Cas9 technology has been applied to many other fungi, such as *Colletotrichum gloeosporioides* (Guo and An

2020), *Fusarium oxysporum* (Wang et al. 2018), *Monilinia fructicola* (Zhang et al. 2020), *Sclerotinia sclerotiorum* (Li et al. 2018), nematode-trapping fungi (Youssar et al. 2019), *Beauveria bassiana* (Chen et al. 2017), and *Blastomyces dermatitidis* (Kujoth et al. 2018).

3. Detection And Treatment Of Fungal Infections

Fungal infections are a serious threat to the health of humans, animals, plants, and ecosystems (Fisher et al. 2012). Every year, deaths caused by fungal infections in humans exceed those caused by tuberculosis or malaria (Brown et al. 2012). Diseases in food crops caused by fungi and decreased yields have become a global food security problem (Bebber et al. 2013). Analyses of the pathogenic mechanism of fungi and improvements in diagnostic and antifungal strategies are urgently needed. CRISPR/Cas technology is expected to revolutionize research on pathogenic fungi in humans and plants.

CRISPR/Cas may be used for the rapid detection and identification of fungi, which is conducive to the early control of fungal infections. Additionally, it can be applied to target virulence genes, while maintaining the viability of fungi, thus preventing infection and avoiding the destruction of the normal flora (Fig 3). It is also a promising method to eliminate drug-resistant genes (Goren et al. 2017), thereby restoring sensitivity to antifungal drugs and reshaping the microbial community. This has been demonstrated in bacteria. For example, Guk et al. (2017) combined CRISPR/Cas9 with DNA fluorescence *in situ* hybridization (FISH) to accurately detect methicillin-resistant *S. aureus* (MRSA). In fungi, Ma et al. (2017) used Cas9-mediated xyloglucan-specific endoglucanase (*PsXEG1*) gene mutation and HDR repair in *P. sojae* to reduce soybean infection rates. CRISPR-Cas9 ribonucleoproteins have been used to target virulence-related genes to combat *M. oryzae*, which seriously damage global rice production (Foster et al. 2018). Zhang et al. (2020) knocked out the *SCRE1* gene of *Ustilaginoidea virens*, thereby significantly reducing its virulence in rice. Schuster et al. (2017) used CRISPR/Cas9 technology to knock out five *EFF1* genes in *U. maydis* at the same time, significantly reducing the virulence of the strain. These studies demonstrate that the CRISPR/Cas system can be used to precisely destroy pathogenic genes in harmful filamentous fungi, providing a new strategy for the prevention and control of fungal diseases.

The CRISPR/Cas system can also be introduced into human pathogenic fungi for targeted intervention. However, methods to transfer gRNAs and Cas proteins with different targets safely and effectively *in vivo* are still needed. At present, delivery methods include physical, viral vector, and non-viral vector methods (Li et al. 2015; Liu et al. 2017; Lino et al. 2018). Physical methods include microinjection, electroporation, and hydrodynamic transfer (Liu et al. 2017). Viral vectors, including adenoviruses and adeno-associated viruses and lentiviruses, can insert genes encoding Cas9 and sgRNA into a single vector for delivery, although concerns about potential carcinogenesis and immunogenicity remain (Li et al. 2015). Non-viral vectors, including lipid nanoparticles and inorganic nanoparticles, have broad potential application because they are relatively safe and easy to package (Li et al. 2015). There is no doubt that CRISPR/Cas technology will promote explorations of the evolution of fungal virulence and host–pathogen interactions and contribute to the development of accurate diagnostic tools and new antifungal drugs.

4. Conclusion And Prospects

CRISPR/Cas is revolutionizing fungal genetic research. In addition to gene knockouts induced by CRISPR/Cas nuclease, a series of distinct optimizations and combinations have been reported, such as CRISPR/Cas9-TAR, CRISPRa/CRISPRi, and CRISPR-Cre recombinase for accurate genome modifications. These tools have unparalleled gene editing ability and are expected to initiate a revolution in fungal research based on CRISPR technology. However, these multi-functional tools still need to be optimized for fungal genome modification. For example, the gene editing efficiency is still very low in macrofungi, and gene manipulation vectors need to be further simplified in yeast and filamentous fungi to improve the knockout efficiency of multiple genes. Using CRISPR technology to target drug resistance genes may be a weapon against fungal drug resistance. For the development of drugs against pathogenic fungi, it is necessary to accurately study the fungal genome, and methods to reduce off-target effects are needed. (Cox et al. 2015; Zhang et al. 2015; Ma et al. 2016; Saha et al. 2019). At present, the Cas nucleases used in fungi are all derived from bacterial Cas9 and Cas12a (Cpf1). It is not clear whether they exert toxicity toward fungal cells or induce an immune response. Many CRISPR systems have not been applied to fungal taxa. It should be noted that DSBs induced by CRISPR/Cas not only activate DNA repair but also cause unpredictable and unnecessary genomic changes, such as random insertions of DSB sites, chromosomal translocations, and large fragment deletions (Kosicki et al. 2018). CRISPR/Cas is expected to have increasingly broad applications for basic theoretical research on fungi, biological resource development, drug development, and other fields, with far-reaching impacts.

Declarations

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Conflict of interests

The authors declare no competing interests.

Author contributions

Biao Ren and Xuedong Zhou contributed to the conception of the study. Binyou Liao and Yujie Zhou contributed to data collection, interpretation and final approval of data for the work. Binyou Liao and Lei Cheng developed the first and final draft of the manuscript. Yangyang Shi and Xingchen Ye developed the second draft of the manuscript. All figures and tables were designed and checked by Ziyi Zhou and Min Liao. Lixin Zhang and Biao Ren critically reviewed and revised the manuscript.

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Tables

Table 1. Summary of the CRISPR/Cas established in fungi

Fungi	Species	Cas proteins	References
Yeast	*S. cerevisiae*	Cas9/Cas12a	(DiCarlo et al. 2013; Li et al. 2018)
	P. kudriavzevii	Cas9	(Sun et al. 2020)
	Y. lipolytica	Cas9/Cas12a	(Schwartz et al. 2017; Yang et al. 2020)
	S. pombe	Cas9/Cas12a	(Jacobs et al. 2014; Zhao and Boeke 2020)
	C.albicans	Cas9	(Vyas et al. 2015)
	C. glabrata	Cas9	(Enkler et al. 2016)
	C. parapsilosis	Cas9	(Lombardi et al. 2017)
	C. neoformans	Cas9	(Wang 2018)
	C. lusitaniae	Cas9	(Grahl et al. 2017)
	C. auris	Cas9	(Rybak et al. 2020)
	C. orthopsilosis	Cas9	(Lombardi et al. 2019)
	C. metapsilosis	Cas9	(Lombardi et al. 2019)
	C. tropicalis	Cas9	(Zhang et al. 2019)
	C. aaseri	Cas9	(Ibrahim et al. 2020)
Filamentous fungi	*T. reesei*	Cas9	(Liu et al. 2015)
	N. crassa	Cas9	(Matsu-Ura T 2015)
	A. oryzae	Cas9	(Katayama et al. 2015)
	A. niger	Cas9/Cas12a	(Kuivanen et al. 2016; Vanegas et al. 2019)
	M. oryzae	Cas9	(Foster et al. 2018)
	M. thermophila	Cas9/Cas12a	(Liu et al. 2017; Liu et al. 2019)
	A. nidulans	Cas9/Cas12a	(Nødvig et al. 2015; Vanegas et al. 2019)
	U. maydis	Cas9	(Liu et al. 2017)
	M. circulalloides	Cas9	(Nagy et al. 2017)
	P. sojae	Cas9	(Miao et al. 2020)
	A. fumigatus	Cas9	(Fuller et al. 2015)
	P. Chrysogenum	Cas9	(Pohl et al. 2016)

	T. atroroseus	Cas9	(Nielsen et al. 2017)
Macrofungi	*A. bisporus*	Cas9	(Waltz 2016)
	G. lucidum	Cas9	(Qin et al. 2017)
	C. militaris	Cas9	(Chen et al. 2018)
	S. bambusicola	Cas9	(Deng et al. 2017)
	C. cinerea	Cas9	(Sugano et al. 2017)
Other fungi	*C. gloeosporioides*	Cas9	(Guo and An 2020)
	F. oxysporum	Cas9	(Wang et al. 2018)
	M. fructicola	Cas9	(Zhang et al. 2020)
	S. sclerotiorum	Cas9	(Li et al. 2018)
	Nematode-trapping fungi	Cas9	(Youssar et al. 2019)
	B. bassiana	Cas9	(Chen et al. 2017)
	B. dermatitidis	Cas9	(Kujoth et al. 2018)

Figures

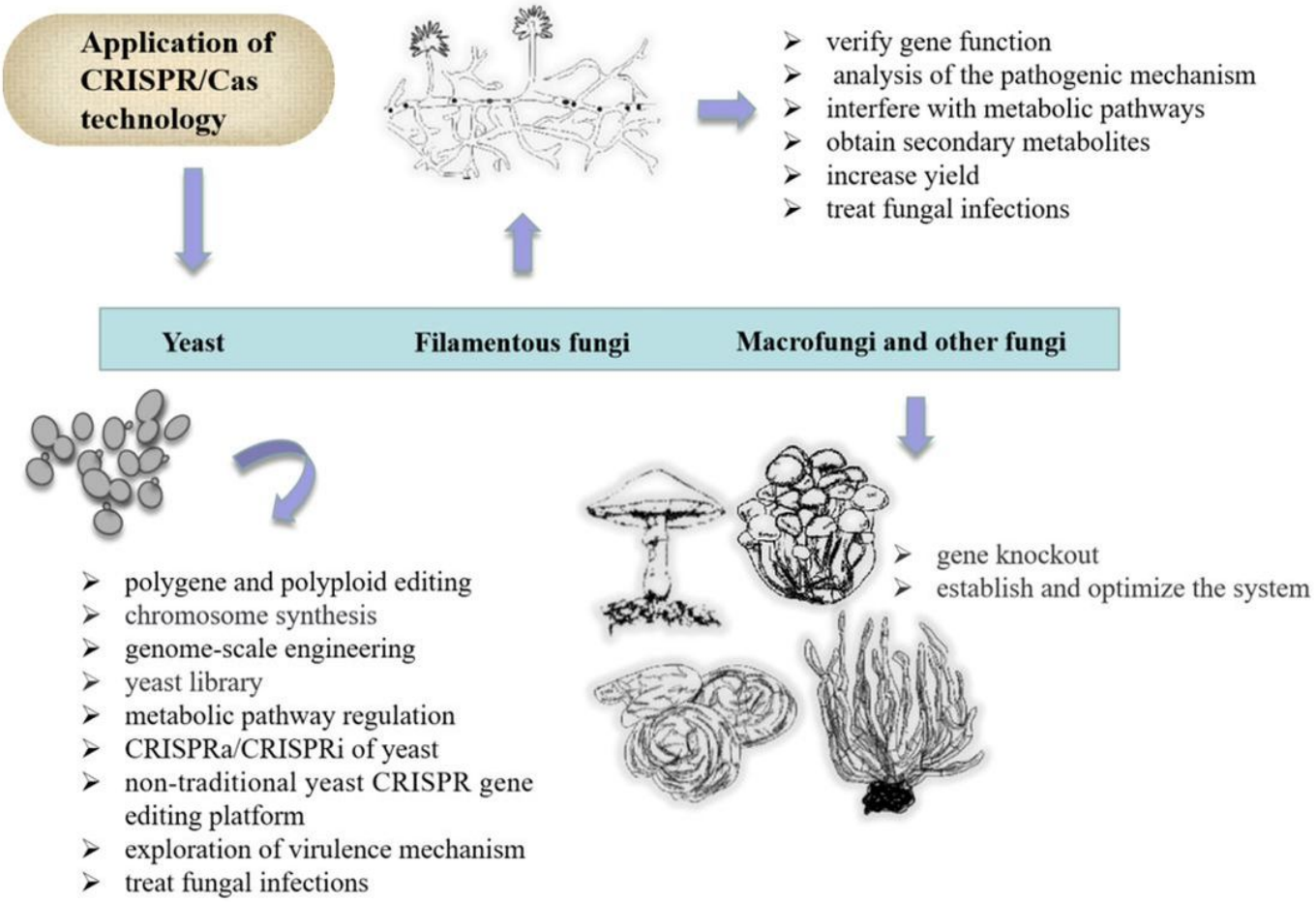

Figure 1

The main application of CRISPR/Cas gene-editing technology in various fungi

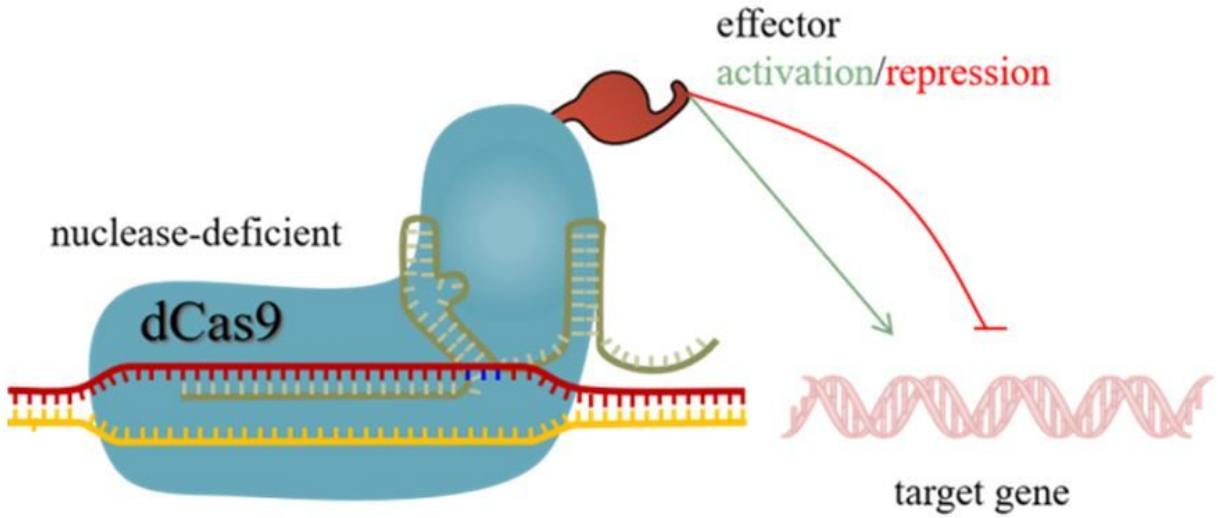

Figure 2

CRISPR activation (CRISPRa) /interaction (CRISPRi)

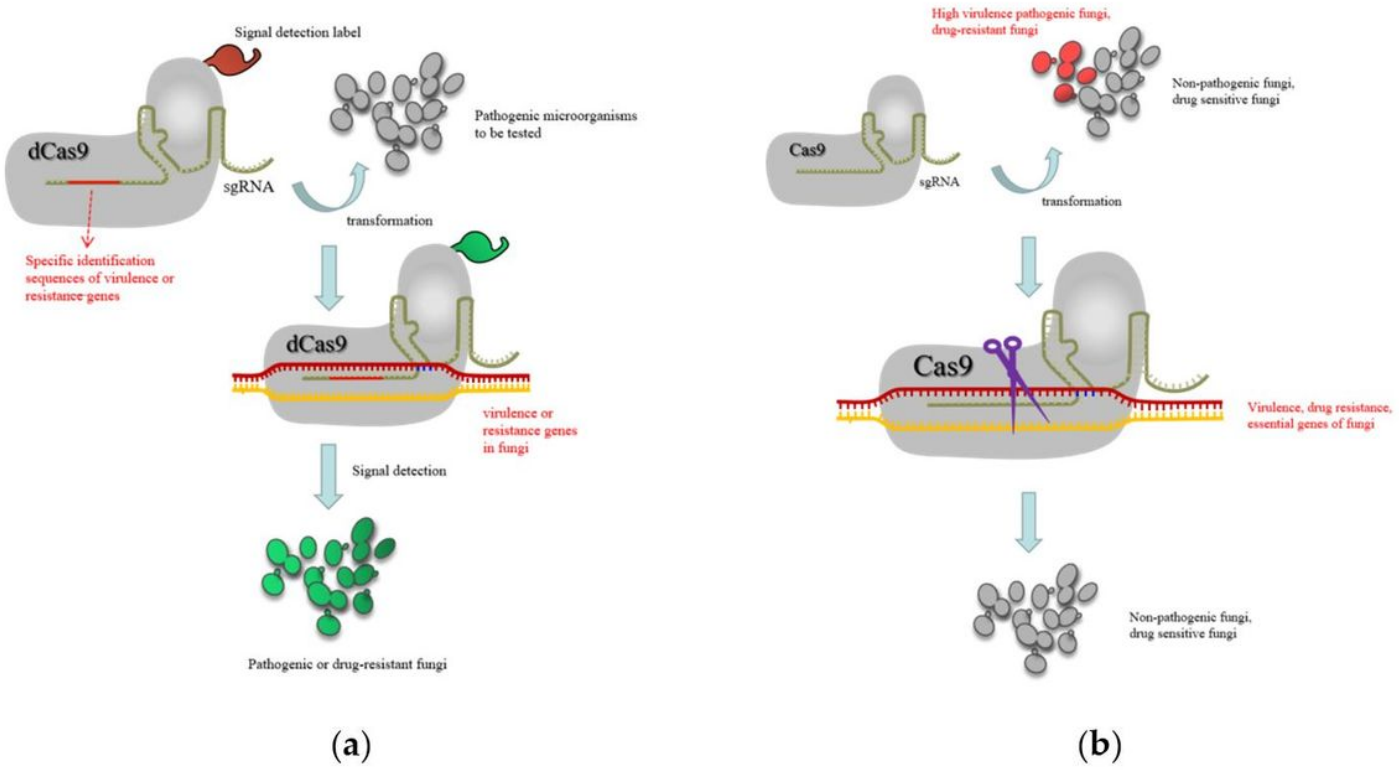

Figure 3

Detect and treat fungal infections. (a) CRISPR used for rapid detection and identification of fungi. The dCas-sgRNA complex with signal tags can identify and combine the specific sequences of fungal virulence or drug resistance genes in pathogenic microorganisms to be tested, so that fungi can be rapidly detected and identified by detecting signals. (b) CRISPR to treat fungal infections or eliminate drug-resistant fungi. The Cas-sgRNA complex identifies and binds virulence genes of fungi, aiming to transform highly virulent pathogenic fungi into non-pathogenic fungi, or target drug-resistant genes to restore the susceptibility of fungi to drugs, or even target some essential genes to kill pathogenic fungi directly.