===== REVIEW =====

CRISPR/Cas Genome Editing in Filamentous Fungi

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Abstract—The review describes the CRISPR/Cas system and its adaptation for the genome editing in filamentous fungi commonly used for production of enzyme complexes, enzymes, secondary metabolites, and other compounds used in industrial biotechnology and agriculture. In the second part of this review, examples of the CRISPR/Cas technology application for improving properties of the industrial strains of fungi from the *Trichoderma*, *Aspergillus*, *Penicillium*, and other genera are presented. Particular attention is given to the efficiency of genome editing, as well as system optimization for specific industrial producers.

INTRODUCTION

For centuries, filamentous fungi have been used by humans for production of useful metabolites and enzymes. The advantage of fungi over other industrial microorganisms is their ability to secrete large amounts of proteins (up to 120-150 g/liter) using relatively simple, cheap, and easily scaled fermentation schemes. Because of their genetic diversity, filamentous fungi can serve as sources of new genes coding for enzymes with unique properties, as well as in the development of recombinant strains for heterologous expression of industrially important proteins [1, 2].

Filamentous fungi commonly used in biotechnology belong to the genera *Aspergillus* [3-5], *Trichoderma* [6, 7], *Penicillium* [8-10], *Acremonium* [11], etc. Enzymatic preparations produced by the industrial fungal strains are used in the production of organic acids, biofuel, antibiotics, biologically active supplements, food products, and fodder. Fungal enzyme preparations are commonly added to detergents and used in the textile, paper, and waste management industries [12-15].

The main objectives in biotechnology involve identification of new enzymes, improvement of their operational stability, development of highly productive strains, and their metabolic engineering. Recombinant DNA technology is used to solve these problems worldwide [4]. Development of optimal expression systems for each strain of filamentous fungi with adapted transformation method are at the core of the recombinant DNA technology. In the case of fungi, the development of expression system involves creation of the recipient strain with dominant or auxotrophic trait for selection and construction of integration plasmid for the transfer of exogenous DNA to the fungal protoplasts [16]. As a rule, heterologous genes are placed under control of strong inducible promoters, i.e., those providing maximum levels of the synthesized protein in the culture liquid of the recipient strain [17]. The *cbhI* promoter in *Trichoderma reesei* and *Penicillium verruculosum* [18-22], *glaA* promoter in *Aspergillus niger* and *P. verruculosum* [19, 20], *xylA* promoter in *Penicillium canescens* [8], and *alcA* promoter in *Aspergillus nidulans* [21] are examples of the strong promoters worth mentioning. When these promoters are used in the optimized fermentation schemes, content of the target heterologous protein can reach up to 50-80% of the total secreted protein [22].

However, even the optimized procedures of heterologous expression in the industrial superproducer strains

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Abbreviations: CRISPR/Cas, clustered regularly interspaced short palindromic repeats and CRISPR-associated protein; DSB, double-strand break; HDV, hepatitis D virus; HH, hammerhead; HR, homologous recombination; NHEJ, nonhomologous end joining; NLS, nuclear localization sequence; sgRNA, single guide RNA; tracrRNA, *trans*-activating CRISPR RNA.
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do not always solve the problems that emerge during expression optimization, such as inhibition of microbial proteolytic activity in order to prevent proteolysis of the heterologous proteins [23], reduction of the mycotoxin content and generation of safer strains [24], development of new auxotrophic strains for subsequent transformation and selection, etc. [25]. Other issues in the transcriptional regulation of heterologous expression essential for industrial application are reduction of the catabolic repression in industrial strain [26, 27], identification of transcriptional activators [28, 29], and optimization of promoter regions involved in heterologous expression [30-32]. Increase of the efficiency of homologous recombination is another important task [33-36]. Finding solutions for these problems will allow to increase productivity of the recombinant strains and efficiency of the technological processes in general due to reduction of expenses associated with the synthesis and purification of enzyme preparations [37].

Until recently, approaches for the strain improvement had been limited to the classic methods of random mutagenesis [38, 39], directed evolution [40, 42], and plasmid development for deletion/substitution of the target genes [43], followed by transformation and selection of the recombinant clones. Random mutagenesis increases the chance of additional unwanted mutations in the fungal genome (especially when several rounds of mutagenesis are performed) that often slow down fungal growth, impair sporulation, and induce genomic instability [44]. Directed evolution also involves the stage of random mutagenesis to generate libraries of target mutant clones by polymerase chain reaction (PCR) [45]. Main limitation of this method is the necessity for screening of mutant clones and, therefore, is used for the improvement of properties of the particular enzyme rather than the producer strain itself [46-48]. The third method involves inactivation of the undesired gene by deletion/mutagenesis or its substitution with another gene (e.g., antibiotic resistance gene) for providing an additional dominant trait for further selection [43]. However, construction of plasmids for the gene substitution requires cloning of the flanking regions (1500 to 2000 bp long) at the 5′- and 3′-ends of the target gene for efficient homologous recombination, which might be time-consuming if the fungal genome sequence is unavailable.

The recently developed genome editing technology, which allows rapid and precise correction of both prokaryotic and eukaryotic genomes is a promising alternative to the existing methods.

Genome editing is a molecular genetic tool that allows to add, remove, or move DNA fragments in an organism's genome. These manipulations allow to turn off genes determined by the experimenter, introduce new genes into pre-selected loci of the genome, or adjust the gene sequences in the body. [49]. Genome editing in combination with genome sequencing mediated rise of a new direction in genetics — reversed genetics, which studies functions of genes with known nucleotide sequences by analyzing consequences of their knock-out or overexpression in an organism [50]. Genome editing has significantly facilitated genetic studies, as it is much less labor-consuming than the traditional methods used for the search of mutants and their genetic analysis. Besides, genome editing promotes the development of biotechnology methods, because it allows to inactivate selected genes (e.g., those involved in the biosynthesis of toxins [51]) and to alter metabolic pathways for creating super-producer strains or producers of new compounds, etc. [52].

The CRISPR/Cas technology (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats-**C**RISPR **as**sociated) is a new and promising genome editing tool [53]. The first articles on application of the CRISPR system were published in 2013 [54-56]. At present, this technology is widely used in the genetic modification of both prokaryotic and eukaryotic organisms, from bacteria to humans [52].

The year of CRISPR discovery is 1987, when Yoshizumi Ishino and colleagues found unusual sequence repeats in the *Escherichia coli* genome [57]. From 1993 to 2000, Francisco Mojica had identified similar motifs in the genomes of 20 microorganisms [58], although functional significance of these sequences had remained unknown. In 2002, Jansen and colleagues revealed that CRISPR cassettes are flanked by the loci coding for proteins homologous with the nuclease and helicase activities [59]. It was shown in 2007 that CRISPR/Cas systems protect bacteria from bacteriophages [60].

The first studies on the genome editing with the CRISPR/Cas system in filamentous fungi were published in 2015 [61], following experiments on the yeast genome editing [62-64]. At present, the studies on the CRISPR/Cas system optimization for its application in filamentous fungi continue in parallel with the attempts to improve the editing system itself by searching for new and optimizing its already known components. Below, we describe the action mechanism of the CRISPR/Cas system and provide examples of its successful application for the genome editing in filamentous fungi.

CRISPR/Cas SYSTEM: COMPONENTS AND MECHANISM OF ACTION

The CRISPR/Cas system includes genomic CRISPR loci and Cas proteins encoded by the genes flanking the CRISPR loci. A CRISPR locus contains direct DNA repeats connected by short (on average, 20 bp) varying nucleotide sequences (spacers). The spacers are inserted into the genome DNA during an adaptive immune response. Cas proteins are divided into two functional modules: the more conserved adaptation module
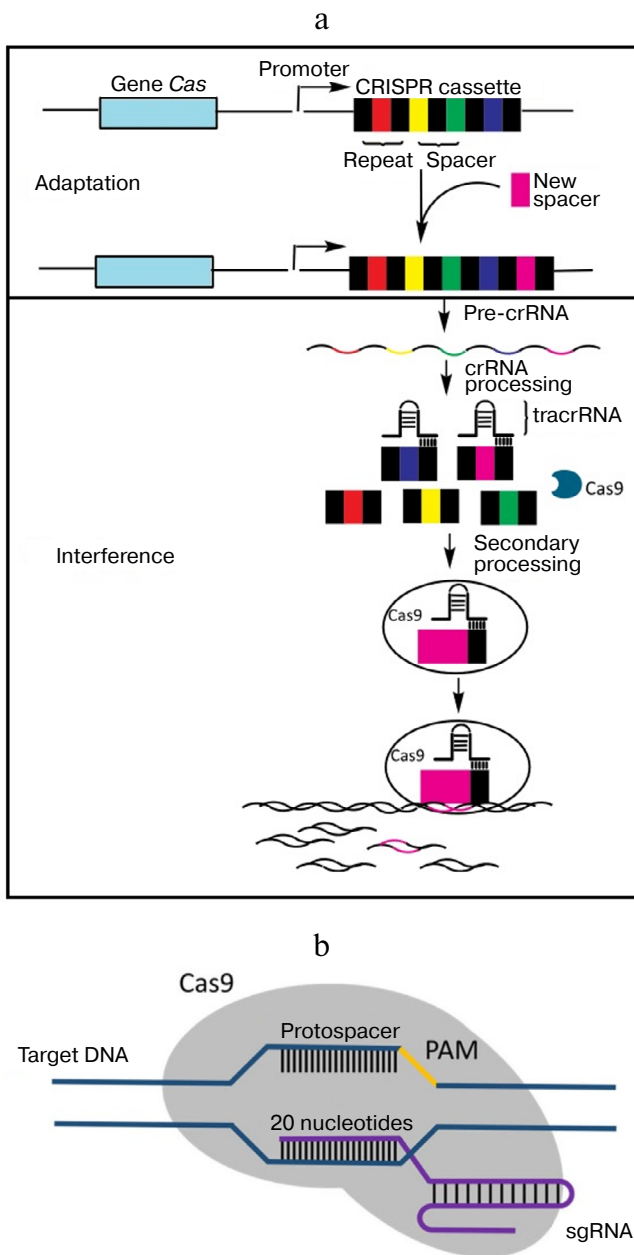
a

b

Fig. 1. a) Principle design of the CRISPR/Cas system. DNA fragment is incorporated as a new spacer in the CRISPR cassette after the first attack. When the same DNA enters the cell again, the spacer is expressed in the crRNA-tracrRNA complex and directs Cas9 to the corresponding DNA for its cleavage. b) CRISPR/Cas system type II. sgRNA binds to Cas9 and catalyzes DSB formation in the DNA targeted by the 20-nt protospacer. (Colored versions of Figs. 1-4 are available in online version of the article and can be accessed at: https://www.springer.com/journal/10541)

and the variable interference module (effector complex). Proteins of the adaptation module provide insertion of new spacers into the CRISPR cassette, while effector complex protein are involved in degradation of the DNA or RNA molecules containing fragments complementary to the spacer sequence (Fig. 1a) [65].

There are two classes of the CRISPR/Cas systems: class 1 systems involve multisubunit effector complexes, while the effector complexes of the class 2 systems use a single type of Cas protein. Each class is subdivided into several types and subtypes based on the presence of a "signature" protein found almost exclusively in this category [66]. Effector complexes currently used for genome editing are mostly of type II because they are easier to use (Fig. 1b) [67].

Despite the significant differences in the composition of effector complexes in microbial species, the mechanism of action of the CRISPR/Cas system is the same. First, phage genomes sequences, mobile elements, or fragments of the cell DNA formed during DNA repair or transcription termination are inserted into the CRISPR cassette. The process of interference starts with transcription of the CRISPR cassette with formation of immature pre-CRISPR RNA (pre-crRNA) composed of multiple repeats and spacers. The primary transcript is processed with generation of the short CRISPR RNAs (crRNAs), each containing a spacer flanked by the fragments of repeats. crRNAs form the effector complex with Cas proteins, which recognizes its target via interaction of the crRNA spacer with the complementary sequence in the foreign DNA (protospacer). This recognition induces hydrolysis of the phosphodiester bond between the bases in the target sequence and degradation of the latter. If the target sequence is present in the cell genome, this might result in the cell death (Fig. 1a) [68, 69].

Type II CRISPR/Cas systems. Based on the differences between Cas proteins, the class 2 CRISPR/Cas systems are classified into 3 types (II, V, VI) [70, 71]. Unlike type I, III, and IV CRISPR/Cas systems (which utilize complex mechanisms of Cas protein interactions), type II CRISPR/Cas system containing Cas9 protein from *Streptococcus pyogenes* is simple, which makes it easy to use for editing microbial genomes [66, 72]. The effector complex of type II systems contains only two components − Cas9 nuclease and single guide RNA (sgRNA) consisting of the fragment that recognizes DNA target (crRNA) and auxiliary *trans*-activating RNA (tracrRNA) that forms hydrogen bonds with crRNA and interacts with Cas9 [73, 74]. The 5′-end of crRNA contains a spacer that determines its specificity toward a particular genomic DNA locus (Fig. 1b) [75, 76].

Structure and function of Cas9 from Streptococcus pyogenes. Cas9 from *S. pyogenes* (SpCas9) is a type II protein from the class 2 effector complexes. It is a multidomain, multifunctional protein composed of 1368 amino acid residues. The 3D structure of SpCas9 in complex with DNA and sgRNA was solved by X-ray diffraction analysis and cryo electron microscopy (Fig. 2) [77]. SpCas9 consists of the nuclease (NUC) and recognition (REC) segments. The NUC segment is composed of the HNH-like and RuvC-like nuclease domains and the C-terminal domain (CTD) that interacts with the protospacer adja-
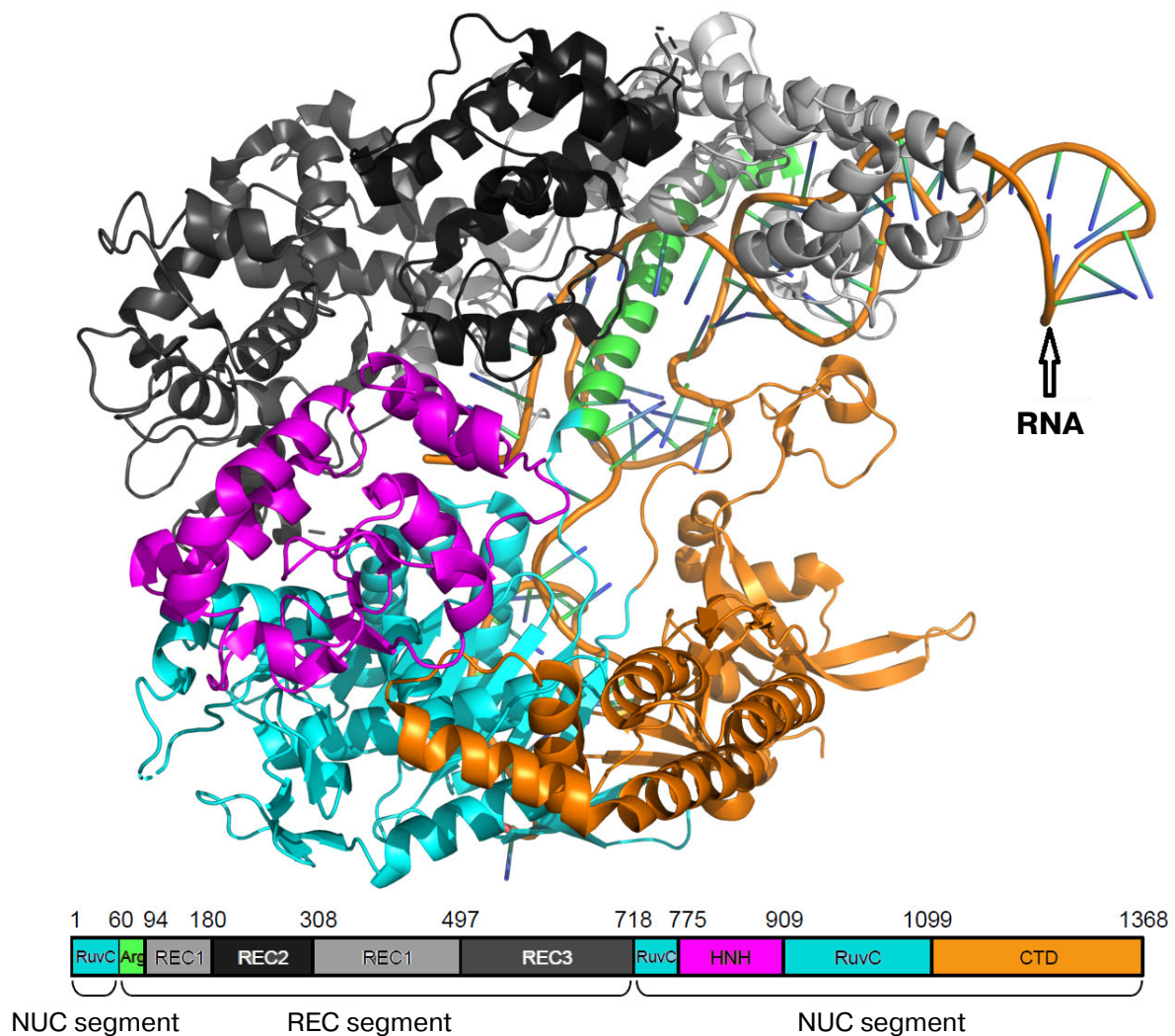
Fig. 2. 3D structure of SpCas9 in the complex with sgRNA (based on the data of XRD analysis; PDB:4ZT9). Lower panel, SpCas9 domain structure.

cent motif (PAM). The REC segment includes three α-helical domains (REC1-3) (Fig. 2).

After the SpCas9/sgRNA complex binds to DNA, the HNH domain cleaves the DNA strand complementary to the sgRNA protospacer, while the RuvC-like domain cleaves the other strand. The Cas9 protein globule completely surrounds the DNA molecule, which enters the tunnel between the CTD and REC1 domain, where it is separated into two strands. One strand binds in the narrow cleft between the RuvC-like domain, HNH domain, and CTD; the other strand forms heteroduplex with sgRNA in the space between the REC and NUC segments. After circumventing the RuvC-like and HNH domains, the DNA duplex reforms behind the protospacer end opposite to the PAM [77, 78].

To facilitate genetic engineering procedures, it was suggested to use common sgRNA (Fig. 3). The 5′-end of this sgRNA is a variable sequence (spacer) of 20

nucleotides (nt); the 3′-end is a constitutive fragment (76 nt) required for Cas9 binding. sgRNA nucleotides 21-41 are part of the native crRNA, while the rest of the molecule is part of the tracrRNA. All fragments of sgRNA bind to the corresponding domains in Cas9, except the spacer and nucleotides 29-59 that form a loop due to complementary interactions between the repeat and anti-repeat. The loop protrudes from the Cas9 protein globule [73].

According to the biochemical and biophysical data, DNA recognition and cleavage by Cas9/sgRNA occurs in several steps. First, sgRNA forms a complex with the Cas9 apoenzyme. The active complex randomly (via 3D diffusion) binds to the DNA. If DNA lacks PAM at the binding site, the complex rapidly dissociates from the DNA and continues "search" for the target. Inability of Cas9 to function via processive mechanism (sliding along the DNA molecule) typical for many other DNA proteins
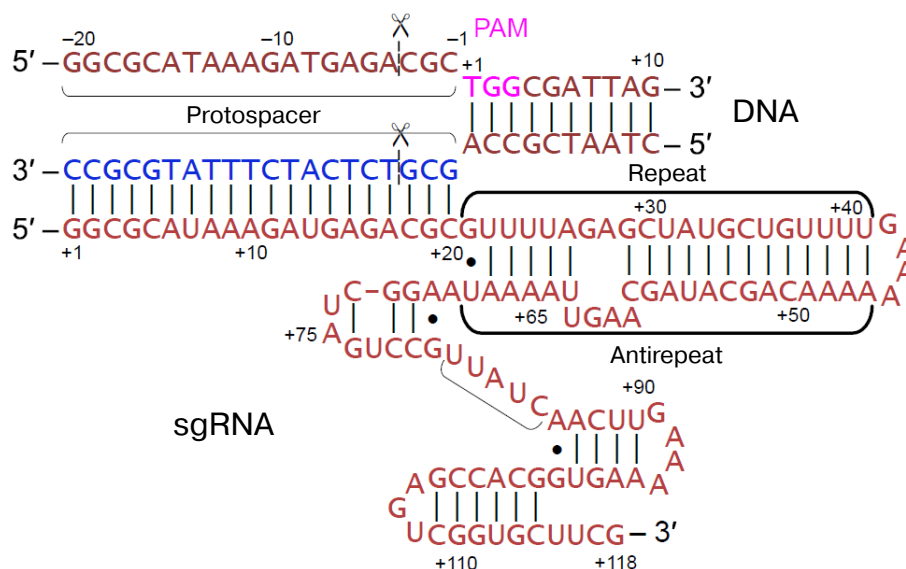
Fig. 3. Secondary structure of sgRNA interacting with the target DNA sequence.

exhibiting affinity to certain sequences, increases significantly (by several orders of magnitude) the time required for the target location and recognition in large eukaryotic genomes [79].

Recognition of PAM in the DNA strand by the Cas9/sgRNA complex is followed by separation of the DNA strands and inspection of the seed sequence [80]. At the same time, the C-terminus of Cas9 stabilizes the phosphate group between the last deoxyribonucleotide of the sequence complementary to the sgRNA protospacer and the first PAM deoxyribonucleotide. As a result, the DNA strand complementary to the protospacer rotates toward the sgRNA for the formation of complementary interaction, while Cas9 stabilizes DNA in the molten state by interacting with the sugar-phosphate backbone in the seed part of the non-target strand [81]. This leads to formation of the RNA protospacer/DNA heteroduplex, whose stability is critical for the target sequence identification. If the DNA sequence is not complementary to the sgRNA in the first eight nucleotides following PAM, the protospacer does not bind to DNA, which prevents interaction between the DNA and the Cas9/sgRNA complex. However, if formation of the heteroduplex occurs in the case of nonspecific binding to the protospacer, the off-target effects in genome editing are possible. Heteroduplex formation induces conformational rearrangements in the Cas9 molecule, resulting in its activation. The catalytic mechanism of the phosphodiester bond hydrolysis is poorly understood. It is only known that the HNH domain acquires its stable conformation after formation of the complete heteroduplex that results in significant conformational changes in the linkers targeting the non-target strand for cleavage in the catalytic site of RuvC [82]. The non-target stand is cleaved by

exonucleases in the $3' \rightarrow 5'$ direction after hydrolysis, while the target strand is degraded [73]. Cas9 remains bound to the cleaved DNA until it is replaced by other enzymes, such as DNA or RNA polymerases. Next, the double-strand breaks (DSBs) can be repaired via two mechanisms: (i) non-homologous end joining (NHEJ) with possible emergence of deletions or insertion at the site of DNA break and (ii) homologous recombination (HR) via insertion of the donor DNA flanked by the homologous sequences, which might be used for insertion of the target gene.

Expression of the Cas9 nuclease for genome editing in filamentous fungi. Expression of the Cas9 nuclease is an important element in optimization the CRISPR/Cas system for the genome editing in filamentous fungi. The first step in the SpCas9 adaptation to the expression in fungi is optimization of the *spCas9* codons with respect to the codon frequency in fungi [61, 83]. Another important issue is selection of the nuclear localization sequence (NLS). The most commonly used NLS is the SV40 sequence [61]; however, in some fungi, e.g., *Fusarium oxysporum*, this typical NLS cannot be used, so homologous NLS sequences are employed [84].

To detect expression and to assess intracellular localization of the optimized Cas9, most researchers use fusion constructs composed of Cas9 and eGFP (enhanced green fluorescent protein), so that nuclear or cytosolic location of Cas9 can be recorded by fluorescence microscopy [84-87].

Cas9 expression also strongly depends on the promoter used. Constitutive promoters are most commonly employed, since they provide stable and induction-independent expression of heterologous genes. The three promoters used for the Cas9 expression in filamentous fungi

are the *trpC* promoter of the tryptophan synthesis gene from *A. niduans*, *gpdA* promoter of the glyceraldehyde 3-phosphate dehydrogenase gene from *A. nidulans* and *P. verruculosum*, and TEF1 promoter of the translation elongation factor 1 α-subunit from *A. nidulans* [61, 88-89]. The less common promoters, which are still used for the Cas9 expression in fungi are *xlnA*, *Ham34*, *amyB*, *niiA*, *Otef*, and *hsp70* [90-93]. It should be noted that heterologous expression of Cas9 may be toxic for the recipient strain; hence, it is important to study its effect on standard morphological and physiological parameters (strain growth and development, sporogenesis, stress resistance, virulence, etc.) during each adaptation of the CRISPR/Cas system in a filamentous fungus strain [87, 94-96]. The data on the developed systems for the Cas9 expression in fungi are summarized in table.

Use of SpCas9 orthologs and other Cas proteins in genome editing. The widespread use of SpCas9 can be likely explained by the fact that this protein was the first discovered second class nuclease. Although SpCas9 has been studied in detail and is widely applied in genome editing, the search for new effector proteins continues. Many Cas9 orthologs exceed SpCas9 in their properties. For example, the proteins from *Streptococcus thermophiles* (StCas9) and *Neisseria meningitides* (NmeCas9) are more specific. The Cas9 gene from *Staphylococcus aureus* is shorter, which facilitates its delivery to the edited organism. In addition, the Cas9 orthologs have different PAM sequences that allow editing different DNA sites and, in some cases, reducing the number of off-target deletions in the edited genome [97].

In addition to the Cas9 proteins, the Cas12a protein (Cpf1) from *Franciella novicida*, which belongs to the class 2 CRISPR/Cas type V system, has been widely used for genome editing. Its advantages include smaller size (1300 amino acids vs. 1629 in SpCas9) and smaller sgRNA (40 nt vs. 100 nt in the SpCas9 sgRNA). Also, the PAM for Cpf1 is located upstream of the protospacer and has the 5′-TTN-3′ sequence, which expands possibilities of the method in general. The cleavage takes place after the 18th nucleotide from PAM in the non-target strand and after the 23rd nucleotide in the target sequence resulting in the formation of 5′-protruding sticky ends [98]. The first article on the successful use of Cpf1 for genome editing in *Saccharomyces cerevisiae* was published in 2017 [99] confirming that Cpf1 can be a promising protein for the genome editing in yeast and other fungi.

Cas13a is an RNA-guided RNA ribonuclease of the type VI-A CRISPR/Cas system that degrades invasive/viral RNAs targeted by crRNA [100]. The crystal structure of Cas13a from *Leptotrichia buccalis* was resolved, and its mechanism of action was elucidated. It was found that Cas13a stays inactive in the absence of viral RNAs complementary to the targeting sequence. However, following phage attack the host and the targeting sequence of crRNA forms a duplex with the RNA-guided target, Cas13a switches to the catalytically active state and randomly cleaves all collateral and single-stranded RNA targets exposed by the ssRNA. These studies demonstrated how the type VI CRISPR/Cas systems containing Cas13a protect cells from the RNA-containing phages and can serve as a basis for developing tools for RNA manipulation.

sgRNA expression. RNA polymerase III promoters. sgRNA in the CRISPR/Cas9 system lacks the cap and the polyA tail and requires clear transcription initiation start. In most cases, stable transcription of sgRNA is guided by the *U6* promoter of RNA polymerase III [101]. To investigate if the *U6* promoter can be used in filamentous fungi, snRNA (small nuclear RNA) sequences from various organisms (from yeast to humans) were compared. Bioinformatic analysis revealed high degree of homology (65%) between the snRNA *U6* sequences of all eukaryotes [91]. Identified *U6* promoters successfully controlled sgRNA transcription and directed Cas9 to the DSB site [92, 94].

The authors of [93] tested efficiency of the CRISPR/Cas9 systems controlled by the tRNA promoters (p*tRNA*Leu-TAA, p*tRNA*GlyGCC, p*tRNA*TyrGTA, and p*tRNA*GlyTCC). All four tRNA promoters were more efficient than the *U6* promoter, indicating the possibility of their application in an alternative expression systems [102].

The search for more efficient promoters resulted in the discovery of a highly conserved region of the 5S rRNA gene. The use of this fragment and 328-nt region directed toward the 5′-end as an sgRNA promoter increased the efficiency of DNA cleavage up to 96% (vs. 25% in the case of the *U6* promoter) [103].

RNA polymerase II promoters. The use of *U6* and *U3* promoters has some limitations. Firstly, *snRNA U6* and *snRNA U3* are constitutive genes expressed almost in all cell types, therefore, they cannot be used for a tissue-specific expression of sgRNAs. Secondly, the *U6* and *U3* promoters cannot be used for *in vitro* sgRNA transcription, because RNA polymerase III is commercially unavailable. Besides, variety of the spacers compatible with the *U6* and *U3* promoters is limited to the $G(N)_{20}GG$ and $A(N)_{20}GG$ sequences, respectively [101, 104].

The abovementioned limitations can be avoided by using ribozymes, which are RNA molecules with catalytic activity. The authors of [102] used the 5′-HH (hammerhead) and 3′-HDV (hepatitis D virus) ribozymes with nuclease activity for the cassette design. The resulting construct was introduced into yeast cells under the control of the *ADH1* promoter transcribed by RNA polymerase II. In this case, the CRISR/Cas9 system was not limited to the use of the $G(N)_{20}GG$ and $A(N)_{20}GG$ sequences. Later, an analogous construct using the strong constitutive *gpdA* promoter and the *trpC* terminator was obtained and successfully tested in *Aspergillus* strains [83].

Structural elements of the CRISPR/Cas9 system for genome editing of lower fungi

Microorganism	sgRNA strategy	Cas9 strategy	Deleted trait/effect	Editing efficiency	References
			Genome editing of *Aspergillus* fungi		
A. aculeatus; *A. brasiliensis*; *A. carbonarius*; *A. lichuensis*; *A. nidulans*; *A. niger*	*in vivo* expression from the same autonomously replicating plasmid: AMA1-P*gpdA*-HH ribozyme-sgRNA-HDV ribozyme-T*trpC*-P*tef1*-Cas9-T*tef1* (NHEJ); optimization of *Cas9* codons for expression in *A. niger*		Δ*albA*/formation of white colonies Δ*yA*/change in the conidium color from green to yellow Δ*pyrG*/growth on 5′-FOA	*A. brasiliensis* and *A. niger* — 100%; *A. luchuensis* — 25% *A. nidulans* — 90% *A. aculeatus* — 80%	[61, 83]
A. oryzae	*in vivo* expression from the same plasmid: P*amyB*-FLAG-NLS-Cas9-NLS-T*amyB*-PU6-sgRNA-TU6-niaD Optimization of *Cas9* codons for expression in *A. oryzae*		Δ*wA*/change in the conidium color from green to white; Δ*yA*/change in the conidium color from green to yellow	10%	[92]
A. oryzae	*in vivo* expression from the same autonomously replicating plasmid: PU6-sgRNA-TU6-AMA1-P*amyB*-FLAG-NLS-Cas9-NLS-T*amyB*		Δ*wA*/change in the conidium color from green to white; Δ*yA*/change in the conidium color from green to yellow; Δ*pyrG*/growth on 5′-FOA	55.6-100%	[134]
A. fumigatis	integration into genome as a single cassette: T*trpC*-*hph*-P*gpdA*-P*tef1*-Cas9-SV40-NLS-P*snr52*-gRNA-T*sup4* (NHEJ); *Cas9* gene codon-optimized for *Homo sapiens*		Δ*pksP*/color change from black to white	25-53%	[114]
A. fumigatis	*in vivo* expression from the same autonomously replicating plasmid: AMA1-PU6-sgRNA-P*gpdA*-Cas9-T*trpC* (MMEJ); optimization of Cas9 codons for expression in *H. sapiens*		Δ*pksP*/color change from black to white	95-100%	[91]
A. niger	*in vivo* expression from the same autonomously replicating plasmid: AMA1-P*tef1*-APOBEC1-XTEN linker-nCas9(D10A)-SGGS linker-UGI-T*tef1*- U6p-sgRNA; the use of editing complex based on nCas9(D10A) and cytidine deaminase		Δ*pyrG*/growth on 5′-FOA; Δ*fwnA*/change in the conidium color from black to white	47-100%	[108]
A. niger	integration into genome as a single cassette: 1) P*gln*-(HDV ribozyme-sgRNA)x4-T*gln*-P*tef1*-Cas9-T*tef1*); 2) P*gpdA*-*amdS*-*gfpE1*-(HDV ribozyme-sgRNA)x4-T*pna2*		Δ4 proteases/41.1-fold increase in erythropoietin production	ND	[131]
A. niger	*in vitro* RNA transcription HR: addition of donor DNA with the marker *pyrG* gene	AMA1-Ptef1-Cas9-*hyg*-T*tef1*	Δ*39114*/4-fold increase in the galactaric acid production	27-100%, depending on the deleted gene of the galactaric acid catabolism	[116]
	RNP complex + donor DNA with the marker *pyrG* gene; transformation by the AMT method	commercially available Cas9 and tracrRNA/crRNA	Δ*gaaX*/60% increase in the galactaric acid production	100%	[128]
A. niger G1 (*amdS*-, Δ*glaA*, Δ*pepA*)	P5SrRNA-HDV ribozyme-sgRNA-6T	P*glaA*-NLS-Cas9-NLS-T*glaA*; optimization of *Cas9* codons for expression in *A. niger*	Δ*albA*/formation of white colonies	100%	[103]

Microorganism	sgRNA strategy	Cas9 strategy	Deleted trait/effect	Editing efficiency	References
Genome editing of *Trichoderma* fungi					
T. reesei Qm6A; *T. reesei* Rut-C30	*in vitro* RNA transcription; addition of donor DNA with the marker *ura5* gene in the case of HR	preliminary integration into genome in the cassettes: P*pdc*-Cas9-eGFP-T*pdc*; P*cbh*I-Cas9-eGFP-T*pdc*; optimization of *Cas9* codons for expression in *T. reesei*	Δ*ura5*/growth on 5′-FOA (NHEJ) Δ*lae1* (HR); Δ*vib1* (HR); Δ*clr2* (HR); Δ*lae1/vib1* (HR); Δ*lae1/vib1/clr2* (HR)	100%; single mutants: 80-90%; double mutants: 45%; triple mutants: 4.2%	[109]
T. reesei Qm6A	*in vitro* RNA transcription; addition of donor DNA with the marker *sxlr* and *ura5* genes in the case of HR; transformation by the PEG/CaCl$_2$ method	preliminary integration into genome as the cassette: P*pdc*-Cas9-T*pdc* (strain from [109])	Δ*sxlr*/selection on media with 1% glucose	ND	[133]
T. reesei	PU61-sgRNA-*ura5*; PU62-sgRNA-*ura5*; transformation by the AMT method T	preliminary integration into genome as the P*pdc*-Cas9-eGFP-T*pdc* cassette: optimization of *Cas9* codons for expression in *T. reesei*	Δ*ura5*/selection on media with 5′-FOA (NHEJ)	8-10%	[120]
T. reesei QM9414 (Δ*pyr4*Δ*mus53*)	*in vitro* Cas9:crRNA stage 1: HR, addition of donor DNA with the marker *pyr4* gene; stage 2: HR, addition of the SES-*cbh1-calB* cassette as a donor DNA		Δ*cbh2*; Δ*egl1*; Δ*egl2*; Δ*cbh2/egl1*; Δ*cbh2/egl2*; Δ*egl1/egl2*; Δ*cbh2/egl1/egl2*	single mutants: 19-23%; double mutants: 6-11%; triple mutants: 12%	[127]
Genome editing of *Penicillium* fungi (*Talaromyces*)					
Penicillium crysogenum	*in vitro* RNA transcription; addition of donor DNA with the marker *amdS* gene	P*Xyl*-hCas9-T*Xyl*-P*gpdA*-*amdS*-T*at*-AMA1	Δ*pks17*/color change from green to white	33-100%	[90]
Talaromyces atroroseus	*in vivo* expression from the same autonomously replicating plasmid: AMA1-P*gpdA*-HH ribozyme-sgRNA-HDV ribozyme-T*trpC*-P*tef1*-*Cas9*-Ttef1; optimization of Cas9 codons for expression in *A. niger*		Δ*albA*/color change from green to white	ND	[110]
Penicillium subrubescens	*in vivo* expression from the same autonomously replicating plasmid: AMA1-P*gpdA*-HH ribozyme-sgRNA-HDV ribozyme-T*trpC*-P*tef1*-Cas9-T*tef1*; optimization of *Cas9* codons for expression in *A. niger*		Δ*ku70*/increased frequency of HR	17%	[111]
Talaromyces pinophilus	*in vivo* expression from the same autonomously replicating plasmid: AMA1-P*gpdA*-HH ribozyme-sgRNA-HDV ribozyme-T*trpC*-P*tef1*-Cas9-T*tef1*; optimization of *Cas9* codons for expression in *A. niger*		Δ*seb1*/short and branched hyphae	43%	[130]

Table (Contd.)

Microorganism	sgRNA strategy	Cas9 strategy	Deleted trait/effect	Editing efficiency	References
Genome editing of fungi from other genera					
Neurospora crassa	P*snr52*-gRNA-T*sup4*	P*trpC*-Cas9-SV40NLS-T*trpC*; optimization of *Cas9* codons for expression in *H. sapiens*	*bar*/cyclosporin A	up to 90%	[88]
Magnaporthe oryzae	integration into genome as the PU6-sgRNA-poly(T)-P*cbh1*-FLAG-NLS-Cas9-T2A cassette		Δ*sdh*/color change from black to pink	up to 90%	[112]
Alternaria alternata	*in vivo* expression from the same autonomously replicating plasmid: AMA1-P*gpdA*-HH ribozyme-sgRNA-HDV ribozyme-T*trpC*-P*tef1*-Cas9-T*tef1*; optimization of *Cas9* codons for expression in *A. niger*		Δ*brm2* and Δ*pks*/loss of colony color	25%	[118]
Acremonium chrysogenum	PU6tRNA-sgRNA; agrobacterial transformation; donor DNA	P*gpdA*-Cas9-T*trpC*; optimization of *Cas9* codons for expression in *A. fumigatis*	Δ*sorB*/loss of the cultural liquid color	90%	[136]
Ustilago maydis	transient expression from the same plasmid: PU6-sgRNA-TU6-P*tef*-NLS-Cas9-HA-NLS-TNOS; optimization of *Cas9* codons		Δ*bW2* or Δ*bE1*/loss of filaments	70%	[94]
Fusarium graminearum	integration into genome as the P*tef1*-HH ribozyme-gRNA-HDV ribozyme-T*gpdA*-P*gpdA*-Cas9-TTRI4 cassette; optimization of *Cas9* codons for expression in *F. graminearum*		Δ*FgOs1*/resistance to DMSO and fludioxonil	ND	[132]

Note. ND, not determined.

The above-described methods are relatively complicated and technically difficult; they can also introduce toxic compounds into the cells. For this reason, researchers have constructed the microRNA-shRNA-sgRNA (miRsh-sgRNA) cassette that was also controlled by the RNA polymerase II promoter [105]. Efficiency of this cassette in sgRNA transcription was proven experimentally using the cleavage of the *p53* gene as a readout [106].

Hence, the RNA II polymerase promoter may be used in future for controlled and safe CRISR/Cas9-mediated editing of genes, including genes in filamentous fungi.

STRATEGIES FOR APPLICATION OF THE CRISPR/Cas SYSTEM IN GENOME EDITING OF FILAMENTOUS FUNGI

The process of genome editing with the CRISPR/Cas system includes: (i) selection of the target gene or locus; (ii) development of the appropriate selection method; (iii) selection of the spacer sequence; (iv) cloning of the sgRNA with the selected spacer; (v) delivery of the CRSPR/Cas system to the edited organism (cell); (vi) selection of the recombinant clones.

The first step is unique for each organism and depends on the purpose of genome editing. The only prerequisite is knowledge of the nucleotide sequence of the edited DNA fragment. So far, very few studies have been devoted to the use of genome editing for metabolic engineering, as the goal of most investigations has been adaptation of the CRISPR/Cas9 system for filamentous fungi [107].

Approaches to selection. Efficiency of the CRISPR/Cas9 system can be easily evaluated by introducing changes in the functional genes in order to alter phenotypic traits of the edited strains (table). Thus, Katayama et al. [92] used the *wA* and *yA* genes as targets to study expression from the *snRNA U6* promoters in the CRISPR/Cas9 system. Deletion of these genes results in the formation of colored conidia. Similar approach was

used in the *Aspergillus* [61, 83, 108], *Trichoderma* [109], *Talaromyces atroroseus* [110], *Penicillium subrubescens* [111], *Magnaporte oryzae* [112], and other fungi (table).

Special attention in the CRISPR/Cas system adaptation is given to the search for efficient selection markers, e.g., antibiotic resistance genes, such as *hph* (resistance to hygromycin B), *ble* (resistance to bleomycin), *bar* (resistance to bialaphos), and *neo* (resistance to geneticin), that have been successfully used for the selection of positive clones. For example, bialaphos-containing medium was used for the selection of *Aspergillus* [61], *N. crassa* [88], and *Myceliophthora* [113] transformants. In [114] and [61], positive clones were selected on the bleomycin-containing medium. Geneticin (G418) was used for the selection of transformed *Phytophthora sojae* clones [115]. Hygromycin B, which is a commonly used selective marker, was applied for the selection of various filamentous fungi, including *A. fumigatus* [114], *A. niger* [116], *A. carbonarius* [117], *Alternaria alternata* [118], and *Shiraia bambusicola* [95].

Orotidine 5-phosphate decarboxylase (PyrG), an enzyme of uridine biosynthesis, is inhibited by 5-fluoroorotic acid (5-FOA). Generation of auxotrophic 5-FAO-resistant strains mutant by the PyrG gene was used for the selection of *A. oryzae*, *T. reesei*, *A. fumigatus* [91, 109, 119, 120]. The *amdS*, gene (coding for acetamidase) ensured the growth of positive *P. chrysogenum* transformants on the medium containing acetamide as a nitrogen and carbon source [90].

However, variety of the selective markers is not limitless because of the individual metabolic features of filamentous fungi. For this reason, the phage Cre recombinase is often used to restore selection markers, so the same marker can be employed for the selection of positive clones. Zhang et al. [121] developed a method for one-step marker-free genetic modification of industrial eukaryotic microorganisms using the CRISPR/Cas9 system based on Cre/loxP. The target gene is knocked-out or deleted by CRISPR/Cas9, while the light-induced Cre recombinase removes selection markers (such as genes providing resistance to hygromycin B, phosphinothricin, and chlorimuron ethyl) to allow the use of the same marker in the following genetic manipulations. The CRISPR/Cas9 system based on Cre/loxP. was successfully used in *Hypocrea jecorina* (*T. reesei*) Qm6a, *N. crassa*, *A. niger*, and *Metarhizium anisopliae* [120]. This system can be also applied for simultaneous removal of several gene clusters via reversible conversion of autotrophs into auxotrophs.

Spacer selection and sgRNA cloning. Selection of the spacer sequence is the most important step, which determines not only efficiency of the genome editing, but also probability of the off-target effects (alterations in the non-target DNA regions). As a rule, the spacer has to satisfy the following requirements: it should not form secondary structures (although some studies used spacers

with the hairpin at the 5′-end to reduce the off-target activity [122]); the GC content should be 35 to 75%; the spacer should lack the poly(T) sequences (when polymerase III is used for sgRNA transcription as they act as termination signals in this case); the off-target sites should contain at least three nonpaired bases, preferably closer to the 3′-end. At present, spacer selection is done with special programs, many of which are open-source programs (E-CRISP: http://www.e-crisp.org/E-CRISP/; CHOPCHOP: https://chopchop.cbu.uib.no/; CRISPRdirect: https://crispr.dbcls.jp/). These programs use various algorithms to estimate the spacer specificity and to search for the possible off-target sites in the edited genome [123].

Cloning of the sgRNA with the selected promoter is a purely technical task comprising a required step in the experimental protocol of the CRISPR/Cas mediated genome editing. There are several approaches for solving this problem (e.g., cloning or PCR), which are beyond the scope of our review. As a rule, cloning involves two steps: annealing of two oligonucleotides with sticky ends corresponding to the spacer sequence with each other and their ligation into the plasmid that had been hydrolyzed by a class IIS restriction endonuclease (e.g., *BbsI*) between the promoter and the sgRNA sequence with formation of the sticky non-complementary ends [103]. This method is universal and allows replacing protospacers for the following genome editing in the particular mycelial fungus.

CRISPR/Cas delivery to the cells. Delivery of the CRISPR/Cas system into the cells of filamentous fungi is the most variable stage in the process of system adaptation for the use in fungi. The system can be introduced into the cells as DNA (with the following *in vivo* assembly), as *in vitro* assembled complex, or in the intermediate state (e.g., as *in vitro* synthesized RNA or sgRNA and the Cas9-encoding DNA). Each method has its own advantages and drawbacks, and the choice for the delivery scheme depends primarily on the edited species and available laboratory resources.

Most often, expression cassettes with the *Cas9* and sgRNA genes are introduced into the edited microorganisms in the content of the same vector or in two separate vectors (table). Zhang et al. [91] investigated efficiency of two different delivery methods using the model fungus *A. fumigatus* and found that the single-vector-based delivery was much more efficient than the delivery with two vectors, which was likely associated with difficulties to control the exact ratio between the *Cas9*- and sgRNA-containing expression cassettes.

The most commonly used delivery method is the step-wise introduction of Cas9 and sgRNA. In the first step, the recipient fungal strain is stably transformed with a cassette carrying the *Cas9* gene, after which the cells are screened for the nuclease expression. In the second stage, these Cas9-positive cells are transformed with the *in vitro*

transcribed sgRNA [85, 109, 116, 124]. Transformation with polyethylene glycol (PEG) and *Agrobacterium*-mediated transformation (AMT) are the two most common methods for the introduction of CRISPR/Cas9 systems into fungal cells. The PEG-mediated transformation is the simplest, so this approach is generally preferred for transformation of numerous fungi [87, 125, 126]. However, the long-term Cas9 expression can be toxic for fungal cells and may promote off-target effects, which could be difficult to identify [62]. Moreover, the use of plasmids for the Cas9 and sgRNA expression could cause random integration of the plasmid DNA into the host genome.

Delivery of the *in vitro* assembled Cas9/sgRNA complexes allows to perform genome editing without incorporation of the *Cas9* and *sgRNA* genes into the host genome, which reduces the possibility of nonspecific editing because of the short lifetime of the complex and keeps the genome "clean". It was shown that the delivery of sgRNA and purified recombinant Cas9 protein to the cells results in the doubling of recombinant *P. chrysogenum* colonies (vs. transformation with a plasmid carrying the Cas9 and sgRNA genes), thus reducing the associated toxicity and off-target effects and providing site-specificity of the introduced mutations [90]. The same study demonstrated that the cell cycle synchronization and timely delivery of the ribonucleoprotein (RNP) complexes of Cas9 represent a simple and efficient method for enhancing the site-specific genome editing in *P. chrysogenum* [90]. Similar methods were used in *T. reesei* [127] and *A. niger* [128]. However, RNP delivery to the cells is challenging and so far is rarely used in filamentous fungi.

Most often, the genome editing of filamentous fungi involves delivery of the Cas9 and sgRNA as the corresponding genes that integrate into the genome or as elements of the plasmid carrying the *AMA1* loci, which provides its autonomous replication during selection [61, 91, 108, 110, 111, 118, 129, 130]. The drawbacks of this approach include the problems with transcription of the sgRNA gene. This gene is commonly placed under control of the RNA polymerase III promoter; however, many species of filamentous fungi lack this promoter. For this reason, researches use the RNA polymerase II promoters and ribozymes to flank the sgRNA sequence [83, 110, 111, 118, 119, 129-132] or employ the conserved 5S rRNA promoter, which is recognized by the RNA polymerase III [103]. The problems with the sgRNA transcription can be avoided by using *in vitro* transcription. However, this requires generation of strains expressing the *Cas9* gene [109].

As mentioned above, edited clones are initially selected based on their phenotype or ability to grow on a selective medium. However, it is often required in practice to edit genes, whose knockouts have no phenotypic manifestations. In this case, a marker gene is introduced

by homologous recombination to the site of cleavage. In any case, the editing event should be confirmed by sequencing after initial analysis of phenotypic changes or selection. If several sites are edited simultaneously, sequencing is the only reliable method for evaluating efficiency of the CRISPR/Cas system.

EXAMPLES OF GENOME EDITING IN FILAMENTOUS FUNGI

Genome editing of *Trichoderma fungi*. *T. reesei* is the major source of commercial preparation of cellulolytic enzymes. This microorganism can be also used for production of heterologous enzymes. The first successful application of the CRISPR/Cas9 system was the knockout of the *lae1*, *vib1*, and *clr2* genes involved in the regulation of expression of cellulase genes. In the first stages of adaptation, codon composition of the *S. pyogenes* Cas9 gene containing the SV40 NLS was optimized, and the codon-optimized *Cas9* gene was integrated into the *T. reesei* genome under control of the constitutive pyruvate decarboxylase (Ppdc) promoter or inducible cellobiohydrolase (Pcbh) promoter. The resulting strains were transformed with the *in vitro* synthesized sgRNA with the spacer corresponding to the target gene. Simultaneously, the marker *ura5* gene was knocked out to provide survival of the transformants on the 5′-FOA-containing medium. The authors demonstrated that the knockout efficiency was much higher when the donor DNA fragments with long (200 bp and over) flanking sequences were used, and that DNA repair via NHEJ was more efficient than via HR [109].

In [133], the procedure developed in [109] was used to knockout the *sxlR* gene coding for the transcription factor negatively regulating the activity of xylanase in *T. reesei*.

In [120], two variants of the promoter region corresponding to the *U6* promoter were cloned for intracellular transcription of sgRNA. The sgRNA contained the protospacer against the marker *ura5* gene. The *T. reesei* cells with basal Cas9 expression were transformed using the AMT method. Efficiency of the genome editing in this case was only 8-10%.

Rantasalo et al. [127] used the nucleoprotein complexes for simultaneous deletion of the cellobiohydrolase 2 (*cbh2*), endogluconase 1 (*egl1*), and endogluconase 2 (*egl2*) genes. The authors transformed *T. reesei* protoplasts with the *in vitro* assembled Cas9:crRNA complex, i.e., used the native crRNA/tracrRNA duplex, rather than *in vitro* transcribed sgRNA. To increase efficiency of HR, the authors used the *T. reesei* strain with mutation in the *pyr4* gene and deleted *mus53* ligase gene. The targeted genes were deleted simultaneously using equimolar amounts of crRNA with different protospacers (two crRNAs per each gene targeted to the start and the end of the gene). The deleted DNA fragments were substituted

with the donor DNA added during the transformation. The donor DNA contained the *pyr4* gene flanked at the 5′- and 3′-ends with homologous 1000-bp sequences. As a result, 16 colonies out of 139 transformants contained the triple deletion (12% efficiency). The same procedure was repeated using the donor DNA cassettes containing synthetic expression system (SES) with the lipase *calB* gene from *Candida antarctica*. The resulting strain produced CaLB lipase preparations with low content of non-target proteins and 1.5-fold higher specific activity compared to the standard expression system using the *cbh1* promoter [127].

Genome editing of *Aspergillus* fungi. Many species of filamentous fungi from the *Aspergillus* genus are widely used in biotechnology for waste treatment, production of organic acids and alcoholic drinks, and other processes. At the same time, some *Aspergillus* fungi synthesize mycotoxin causing diseases in humans and animals.

In 2015, the Uffe Mortensen's group reported the CRISPR/Cas system adapted for genome editing in six *Aspergillus* species: *A. aculeatus*, *A. brasiliensis*, *A. carbonarius*, *A. lichuensis*, *A. nidulans*, and *A. niger* [61]. The genes coding for the CRISPR/Cas system components were delivered to the cells in the content of the same plasmid. In order to be used in different species, four variants of the plasmid were produced that contained different marker genes commonly used in filamentous fungi: *AFUM_pyrG*, *AN_argB*, *ble^R*, and *hyg^R*. The plasmids also carried *AMA1* sequence to ensure its replication. The optimized *SpCas9* gene with theSV40 NLS was expressed in *A. niger* using the strong constitutive promoter of the *A. niger* translation elongation factor 1 gene (*tef1*) and *tef1* terminator. The sgRNA was transcribed under control of the strong *A. niger* glyceraldehyde 3-phosphate dehydrogenase constitutive promoter (*gpdA*) and the *trpC* terminator. Since sgRNA should lack 5′-cap and 3′-poly(A) tail, its sequence was flanked by the HH ribozyme at the 5′-end and HDV ribozyme at the 3′-end (Fig. 4; table).

The developed system was tested with the protospacer against the *albA* gene in five fungal species: *A. aculeatus*, *A. niger*, *A. carbonarius*, *A. luchuensis*, and *A. brasiliensis*. The knockout of the *albA* gene in filamentous fungi results in formation of white colonies. The highest editing efficiency was observed in *A. brasiliensis* and *A. niger* (approximately, 100%); the lowest efficiency was found in *A. luchuensis* (25%), which could be related to a lower survival rate of the transformants. Editing efficiency of the adapted CRISPR/Cas system was verified with the protospacer against the *yA* gene in *A. nidulans* and protospacer against the *pyrG* genes in *A. aculeatus*. The authors concluded that the efficiency of the adapted editing system differed in the fungal species of the same genus; they suggested that these differences could be eliminated by cloning of homologous promoters for the expression of Cas9 and sgRNA in each edited species.
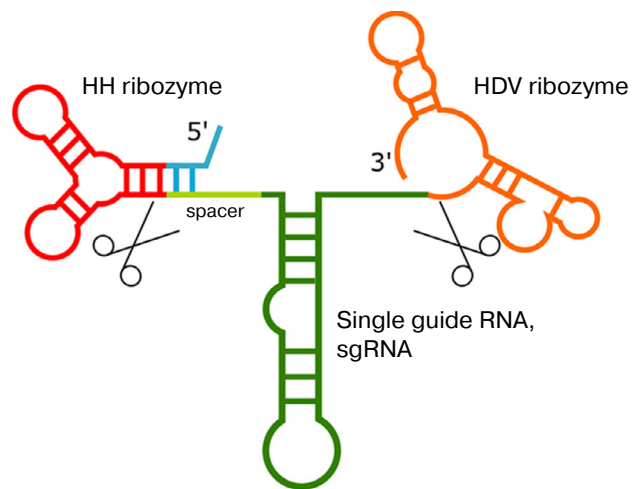

Fig. 4. sgRNA release from the transcript synthesized by RNA polymerase II. Light green and green, sgRNA; red and blue, HH ribozyme; orange, HDV ribozyme; scissors, cleavage site. HH fragment marked with blue color hybridizes with the protospacer for efficient cleavage (adapted from [61]).

Another problem in the editing of filamentous *Aspergillus* fungi was multinuclearity of the protoplasts, which also decreased efficiency of the genome editing [61].

The study of Kuivanen et al. [116] is another example of using genome editing for metabolic engineering. The authors modified the *A. niger* genome to obtain a producer of galactaric acid. First, they sequenced and compared *A. niger* transcriptomes before and after cultivation for 5 and 18 h on a galactaric acid-containing medium in order to identify the genes involved in catabolism of this compound and found that expression of 7 genes was upregulated. Six out of seven of these genes were sequentially knocked out using the CRISPR/Cas system. For this, the corresponding strain was transformed with a plasmid carrying the *Cas9* gene, the *AMA1* sequence, and the *hyg* selective marker for the growth on hygromycin-containing medium. The synthesized sgRNAs (two per gene, targeted to its 5′- and 3′-regions) were added together with the plasmid. The knockout efficiency was 27 to 100%, depending on the deleted gene. In parallel, the same genes were knocked out using deletion cassettes with the homologous 1.5-kb shoulders and *pyrG* gene as a selective marker. In this case, the efficiency of genome editing was significantly less (0 to 43%) [61].

Adaptation of the CRISPR/Cas9B system for *A. niger* performed in [128] included minimization of the *in vitro* protoplast transformation method by the Cas9::sgRNA ribonucleoprotein (RNP) complex. Transformation was carried out in 96-well microplates instead of 15-ml tubes, which significantly reduced the time of procedure and the amount of reagents used. Efficiency of the target gene deletion by this optimized

method was 100%. It was demonstrated that the developed method was suitable for multiplex editing of two or three targets, which is very convenient for the metabolic engineering of fungi. As a result, the authors obtained an *A. niger* strain with enhanced galactarate production.

In 2018, Zheng et al. published a paper on the *in vivo* sgRNA expression in *A. niger* [103]. In this work, the authors used various promoters for the sgRNA expression: human *RNU6-1* promoter (P*h*U6), *A. niger RNU6* promoter (P*an*U6), and *A. niger* 5S rRNA internal promoter with different length of the 5′-untranscribed region (-338, -160, -106, -65, -35) or lacking this sequence. When the 5S rRNA internal promoter was used, the transcript contained the 5S rRNA sequence at the 5′-end; therefore, a construct containing the HDV ribozyme sequence between the 3′-end of 5S rRNA and 5′-end of sgRNA was obtained. To verify efficiency of the selected promoters, *A. niger* G1 (*amdS*-, Δ*glaA*, Δ*pepA*) strain was transformed with the plasmid containing the optimized *SpCas9* gene and plasmids with the sgRNA and the protospacer against the *alba* gene under control of the tested promoters. The knockout of the *albA* gene results in the disruption of melanin synthesis and formation of colorless colonies. The authors found that the efficiency of the P*h*U6 and P*an*U6 promoters was low (20% or less of knockouts from the total number of grown transformants), while all the constructs containing the 5S rRNA promoter have generated knockout in 100% of the transformed colonies. Presence of the 5S rRNA not only failed to decrease knockout efficiency, but even made the procedure more efficient, which could be explained by protection of the 5′-end of sgRNA from RNases.

Next two examples demonstrate possibility of the CRISPR *in vivo* expression from one autonomously replicating plasmid [108, 131]. In [108], the CRISPR/Cas9 system was adapted for editing of the *A. niger* genome in order to prevent possible consequences of DSBs. The authors developed a series of constructs with the modified Cas9 and cytidine deaminase (enzyme converting cytidine into thymine without DSB induction). Two phenotype-associated genes, *pyrG* and *fwnA,* were inactivated with the efficiency of 47.36-100%; the non-phenotype-associated *prtT* gene was inactivated with 60% efficiency [108].

Another case is biotechnological application of the CRISPR/Cas9 system for modification of the recombinant *A. niger* producer strain synthesizing human erythropoietin [131]. Four genes coding for proteolytic enzymes in the *A. niger* rHuEPO strain were knocked-out (*vps*, *prtT*, *algC*, and *och1*) to yield the strain producing no proteases, which significantly (41-fold) increased the level of erythropoietin secretion. The components of the CRISPR/Cas9 system were delivered to the cells in the composition of the same plasmid containing the *AMA1* sequence, which was developed previously for the *Aspergillus* fungi [61].

A. oryzae also belongs to the cellulase-producing industrial filamentous fungi. In Japan, *A. oryzae* is the main microbial producer of homologous and heterologous enzymes. In 2016, Katayama et al. [92] adapted the CRISPR/Cas9 system for *A. oryzae* mutagenesis (10% efficiency). Later, the same authors used the plasmid containing the autonomous replication *AMA1* sequence to increase editing efficiency in *A. oryzae* to 50-100% [134]. Several copies of the *AMA1*-containing plasmid were present in the transformed strains, thereby increasing the level of Cas9 and sgRNA expression and, hence, providing more efficient mutagenesis.

In 2015, Fuller et al. [114] adapted the CRISPR/Cas system for *A. fumigatis* [114] and verified its efficiency for the deletion of the *pksP* gene coding for polyketide synthase responsible for the conidia black color. The CRISPR/Cas system was integrated into the fungal genome as a single cassette; the *Cas9* gene was optimized by using *H. sapiens* codons and linked with the hygromycin resistance gene *hph*. The editing efficiency was low (25-35%), which might be explained by the use of NHEJ prone to generation of random insertions [114].

The authors of [91] attempted to develop a highly efficient (95-100%) CRISPR/Cas system for precise sequence integration into the reading frame (with or without the marker) using very short (~35 bp) flanking regions. This method was called the microhomology-mediated end joining (MMEJ). Using this system, the authors achieved efficient and precise integration of the exogenous GFP into the targeted site without marker insertion. The *pksP* gene providing melanin synthesis in the conidia (see [114]) was used for the clone selection; *in vivo* expression was carried out using the *AMA*-based cassette developed in [61].

Genome editing of Penicillium fungi. Filamentous fungus *Penicillium chrysogenum* is a known producer of β-lactam antibiotics and various secondary metabolites. Successful CRISPR/Cas genome editing of this species was described in [90]. The use of the CRISPR/Cas system was verified using the polyketide synthase gene (*pks17*), knockout of which disrupts biosynthesis of the green pigment in the spores and can be used for the clone selection. The protoplasts were transformed with the *in vitro* synthesized Cas9/sgRNA complex and donor DNA with the acetamidase gene (*amdS*) with 1-kb flanks homologous to the *pks17* gene. The strain with disrupted *hdfA* gene to promote HR was used as a recipient. Correct insertion of the *amdS* gene was confirmed by sequencing. Similarly, the *pks17* and *lovF* genes coding for diketide synthase involved in lovastatin synthesis were knocked out with 50% efficiency. It was also demonstrated that the 60-bp length of homologous shoulders is sufficient for the successful CRISPR/Cas-mediated incorporation of donor DNA in the *P. chrysogenum* strain with disrupted nonhomologous recombination. The authors also transformed the protoplasts with

linear DNA molecules, one of which contained the *amdS* marker and the other – the *AMA1* sequence, *Cas9*, and sgRNA. They suggested that these molecules would undergo *in vivo* recombination due to the existence of homologous ends with the formation of autonomously replicating plasmid with the following selection on acetamide. The *Cas9* gene was placed under the xylose-induced *xlnA* promoter, while sgRNA transcription was studied using three RNA polymerase III promoters (*PtRAN-Met*, *PtRNA-Leu*, and *PU6*). All three promoters provided sgRNA synthesis sufficient for genome editing. However, some clones retained acetamide resistance after several passages on the acetamide-free medium, which suggested incorporation of the marker gene into the fungal genome. The authors concluded based on comparison of the procedures of *P. chrysogenum* genome editing involving the *in vitro* assembled complex and expression of its components from the *AMA1*-carrying plasmid that the first method minimized the off-target effects, while the second method provided higher editing efficiency [90].

A good example of the CRISPR/Cas application in the reverse genetics is the study by Nielsen et al. [110] on modification of biosynthetic pathways for the secondary metabolites in the filamentous fungus *Talaromyces atroroseus*. *T. atroroseus* produces talaroconvolutin A and analogue of the talaroconvolutin B, a compound named ZG-1494α, which is inhibitor of the platelet acetyltransferase activating factor. Since the *T. atroroseus* genome had been sequenced, the authors applied the CRISPR/Cas system earlier developed for *Aspergillus* [116]. They used the donor plasmid carrying the hygromycin resistance gene for HR at the DSB site and three different plasmids with the *AMA1* sequence, *Cas9*, and sgRNA gene, which differed in the protospacer sequences. The UA08_00425 gene presumably coding for the polyketide synthase (enzyme ensuring gray color of the conidia) was used as a target. The authors were able to obtain transformants with colorless conidia, but the efficiency of editing with different protospacers differed by an order of magnitude. Next, the protospacers were selected to knockout the UA08_04451 gene, which encodes an ortholog of the *ccsA* gene required for the synthesis of compounds structurally similar to talaroconvolutin A and ZG-1494α in other fungi. As a result, the clones with the deleted UA08_04451 gene also demonstrated disrupted synthesis of talaroconvolutin A and ZG-1494α [110].

Two studies published in 2020 reported genome editing of *Penicillium subrubescens* [111] and *Talaromyces pinophilus* [130]. In both cases, the authors used the scheme developed for *Aspergillus* [83]. The knockout of the *ku70* gene in *P. subrubescens* increased probability of HR in the absence of phenotypic changes. Although the *ku70* knockout was verified, no increase in the HR frequency was confirmed [111].

The CRISPR/Cas9 was also adapted for the use in *Talaromyces pinophilus*, which is a known producer of carbohydrases. The knockout of the *seb1* gene resulted in formation of the morphologically altered hyphae (shorter and more branched compared to the wild type) and increased enzymes secretion by 10-15%. The cultural liquid of the mutant strains was characterized by higher (by 20-40%) specific activity of cellulases toward filter paper [130].

Genome editing of filamentous fungi from other genera. As is well known, other filamentous fungi are also widely used in biotechnology, for example, *Acremonium chrysogenum* is an industrial producer of cephalosporin. There are also multiple parasitic fungi (*Alternaria alternate*, *Magnaporthe oryzae*, *Fusarium graminearum*) fight against which requires the use of modern methods of molecular genetics. *Neurospora crassa* is a convenient object for studying fungal physiology. There is enough knowledge and experience accumulated in the genome editing in fungi of the *Neurospora*, *Phytophthora*, *Mucor*, *Fusarium*, and other genera [135].

In the study [88], the CRISPR/Cas technology was used to replace the inducible *clr2* promoter (regulator of cellulase biosynthesis) by the constitutive β-tubulin promoter in the genome of *N. crassa* in order to increase the level of cellulase expression. Cas9 and sgRNA were delivered to the cells in two different plasmids. sgRNA was transcribed using the *snr52* promoter and the *sup4* terminator; transcription of the humanized *Cas9* gene was controlled by the *trpC* gene promoter and terminator. The donor DNA was added to replace the *clr2* gene promoter region with the components of the editing complex. The editing efficiency was high (up to 90%) [88].

The CRISPR/Cas was also used for the genome editing in *F. graminearum*, a common crop pathogen. The target gene was *FgOs1*, which codes for the osmosensing histidine kinase. The knockout of this gene results in the resistance to phenylpyrrole and dicarboximide. The components of the CRISPR/Cas system were integrated into the fungal genome as a single cassette; sgRNA transcription was controlled by the *tef1* promoter and *gpdA* terminator separated from the sgRNA sequence by the ribozymes. Transcription of the optimized *Cas9* gene was regulated by the *gpdA* gene promoter and the *TRI4* terminator [132].

Another pathogenic basidiomycete, *Ustilago maydis*, that causes smut on corn and teosinte, was also subjected to genome editing.

The possibility of the CRISPR/Cas system application in this fungus was verified by knocking out the *bE1* and *bW2* genes. The components of the CRISPR/Cas system were transiently expressed in the fungal cells after cell transformation with the plasmid; sgRNA expression was regulated by the *U6* promoter, while the *Cas9* gene was controlled by the *tef1* promoter. No donor DNA was used, and the editing efficiency was 70-100%.

A. chrysogenum C10 is used in the industrial production of cephalosporin C. However, regulation of the cephalosporin biosynthesis is still poorly understood, mostly because of the technical difficulties of genetic manipulations of the industrial strain. In their recent work, Chen et al. [136] constructed an original CRISPR-Cas9 system based on the chimeric *U6/tRNA* promoter. The editing efficiency with this system reached 90%. The authors used donor repair DNAs. The system was tested for the removal of large (up to 31.5 bp) DNA fragments involved in the biosynthesis of yellow compound sorbicillinoid, which allowed easy selection of the recombinant clones based on the color of culture liquid. Interestingly, the knockout strain also exhibited increased synthesis of cephalosporin.

CONCLUSIONS

The CRISPR/Cas system has been successfully adapted and is now commonly used for the genome editing in many filamentous fungi. In the last five years, starting from the first case of genome editing in *T. reesei*, dozens of genomes have been modified in fungal species widely used in industrial biotechnology and agriculture as sources of enzymes, enzyme preparations, secondary metabolites, and other compounds.

One of the major problems in the work with filamentous fungi is the absence of known RNA polymerase III promoters in them. However, this disadvantage can be solved by using either RNA polymerase II promoter and ribozymes or 5S rRNA promoter. It should also be mentioned that the problem of the off-target effects is less urgent in filamentous fungi, since these microorganisms are used for the production of specific compounds. If the off-targets effects do not reduce industrial value of the modified fungus, they can be neglected.

At present, the CRISPR/Cas system still remains an advanced genome editing technology. It is the most universal tool for improvement of physiological and biochemical properties of micro- and macroorganisms. The CRISPR/Cas system is rapidly developing; however, there are still problems related to the editing efficiency, off-target effects, as well as ethical issues associated with genetic modifications. In the nearest future, application of the CRISPR/Cas system in genomics and cell biology will significantly expand the knowledge that has been acquired by using other DNA technologies.

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