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CRISPR/Cas9: Contemporary designer nucleases for efficient genome editing in phytopathogenic fungi

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Abstract

Plant diseases caused by fungal pathogens are one of the main factors contributing to severe economic losses due to reductions in yield and the quality of crops. Studying the fungal genes related to pathogenicity to reveal their infection mechanism through genome editing can play an important role in the management of these diseases. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated nuclease 9 (Cas9) system is a versatile tool for genome engineering which has recently been adopted for sequence specific regulation of gene expression in many plant pathogenic fungal genomes. It is the current scientific consensus point of view that this simple RNA guided genome editing tool is cheaper, easier to use, and is higher in gene modification efficiency than any other available gene editing tool. In this mini review, we discuss the molecular mechanisms underlying the CRISPR/Cas9 technique and its recent improvements and applications beyond gene editing. We discuss and summarize a few recent studies targeting phytopathogenic fungal genomes, potential applications, the remaining challenges, and future perspectives. Our analysis provides insights into how this method can be more widely applied to combat fungal phytopathogens.

Keywords – DNA repair – Fungal genomes – Nucleases – Targeted mutations

Introduction

Plant pathogenic fungi are among one of the most diverse and economically relevant threats concerning plant diseases (Borrelli et al. 2018). Many agriculturally important staple food crops such as maize, rice, wheat and economically important crops such as grape, tea, and many more face significant losses annually due to the devastating effects of fungal diseases (Gramaje & Armengol 2011, Yan et al. 2013, Nalley et al. 2016, Thompson & Raizada 2018). Fungi such as *Blumeria graminis*, *Botrytis cinerea*, *Fusarium graminearum*, and *Pyricularia oryzae* are few examples of fungal pathogens considered to be some of the most important plant pathogens in agriculture (Dean et al. 2012). The emergence of new and more aggressive fungal pathogens has increased substantially since the early 2000s (Fisher et al. 2012), thus developing new control

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strategies for these diseases have become a relevant issue more than ever.

Management of most plant diseases can be easily done if the pathogenicity of the fungus and the host resistance are understood. Fungi employ a wide array of unique mechanisms to colonize a specific plant host to ensure disease establishment and development (Doehlemann et al. 2017). Plant pathology related research has advanced substantially due to the integration of molecular techniques in understanding pathogenesis (McCartney et al. 2003). The rapid development of techniques such as microscopy, DNA, RNA, and protein sequencing combined with bioinformatics has revolutionized the aspects of pathogen detection, understanding disease progression, and has guided new strategies for improving disease resistance (Soanes et al. 2007, Wang & Jin 2017). In order to develop new resistance strategies, in depth understanding of the molecular basis of host pathogen interaction can be very important. Regardless of the improvement in molecular research related to the understanding of plant pathogenic interactions, much more crucial and practically utilizable information remains to be discovered. The technological advancements involving genome sequencing have provided a much-needed platform to bridge this knowledge gap.

Owing to the increase in affordable techniques for whole genome sequencing during the past decade, the number of fungal species with complete genome sequences has significantly increased (Hu 2013). Many independent studies, as well as combined initiatives, have produced whole genome sequences for many fungal species. More than 1500 fungal genomes have been completely sequenced up to now, which is more than plant and animal genomes combined (SOWF: Leitch et al. 2018). The database of the broad institute alone records more than 100 sequenced genomes of different types of fungi including model organisms, human pathogens, and phytopathogens (Broad institute 2019). The convergence of this available wealth of genetic information into functionally and clinically relevant knowledge has always been a major challenge faced by researchers in many fields. Thus, establishing efficient and reliable methods to determine the molecular mechanisms of genes responsible for the relevant and specific phenotypes has become a focal point in molecular research. The best approach in determining the function of a gene is to either shut it down or overexpress it within a living organism; however, this can be very tedious and time consuming (Alberts et al. 2002).

Characterization of genotypic and phenotypic relationships using the loss of function approach is considered the best course of action by molecular biologists for decades (Alberts et al. 2002). Gene knockdowns using RNAi (RNA interference), conventional knockout techniques using homologous recombination, and artificial nucleases with genome modifying or editing ability have been applied in many instances to elucidate the function of a particular gene of interest (Meyer 2008, Sarkari et al. 2017). Conventional knockout methods using homologous recombination and RNAi has been in the frontier of functional genomics as tools that can silence a gene in order to obtain insights into the gene's function (Wang et al. 2017). Homologous recombination was reported to be variably successful and was very cumbersome to perform and gene silencing with RNAi provided a comparatively better alternative (Sen & Blau 2006). Though these techniques were used to elucidate the gene function, the occurrence of false negative results due to inefficient knockdown of the gene, false positive results obtained due to unintentional gene silencing in off-targets, instability of hairpin loops within the target organisms, and the occurrence of hypomorphic phenotypes not mirroring the complete loss of the function of a gene, limited their use (Torres-Martínez & Ruiz-Vázquez 2017). Furthermore, RNAi was reported to be absent in some fungi due to the complete loss of RNAi component related genes during evolution (Drinnenberg et al. 2011, Nicolás et al. 2013). Therefore, using this system for gene knockdown was not possible for some fungal species. Hence, the search for a better tool remained an issue to overcome these limitations.

To overcome the limitations of conventional gene knockout techniques and RNAi, a more efficient approach known as 'genome editing' came into play (Cox et al. 2015). This approach used various artificial nuclease systems to induce changes in the target genomes. While RNAi technology was only capable of regulating post-transcriptional gene expression, the artificial nucleases with genome editing capabilities introduce more permanent changes that can be passed onto the next generation (Boettcher & McManus 2015). Recent studies on functional genomics,

gene therapy and transgenic organisms show successful instances in which genome editing was implemented (Urnov et al. 2010, Wood et al. 2011, Reyon et al. 2012, Friedland et al. 2013). By incorporating engineered, programmable and highly specific nucleases, a cellular organism's genome could be edited by introducing a modification into the site of choice at a predetermined locus via insertion, replacement, or by permanent disruption of the gene. The nucleases induce site-specific changes in the genome through sequence specific DNA binding domains and nonspecific DNA cleavage domains. Insertions, deletions, and substitutions at the loci of interest are generated by different cellular DNA repair processes (Arazoe et al. 2015a). These changes in genes could lead to modifications of the gene function, creating single nucleotide polymorphisms or disease variants and fusion proteins. For example, through the manipulation of candidate virulence genes in phytopathogenic fungi, the gene's relevance to disease development can be determined using these engineered artificial nucleases.

In genome editing, the reprogrammable recognition site is the most important feature in artificial nucleases (Chandrasegaran & Carroll 2016). Zinc Finger Nucleases (ZFNs) (Weinthal et al. 2010, Carroll 2011), transcription activator like effector nucleases (TALENs) (Arazoe et al. 2015b), and RNA guided nucleases (RGNs) in CRISPR/Cas system are being used as genome editing tools (Gaj et al. 2013). Both ZFNs and TALENs function through protein DNA interactions with the use of the modified restriction enzyme *Fok I*, where the nuclease domain of the enzyme is fused with the DNA binding domains of the transcription factors. Even though *Fok I* is programmable and site specific, targeting a new site requires engineering and cloning a new protein which limits its use to one time and excludes being used for high throughput applications. The CRISPR/Cas system has gained more favor from researchers (Wu et al. 2014a) because it depends on a small RNA, not DNA, which leads to its specificity (Ran et al. 2013). The CRISPR/Cas9 system can be used to target multiple genes by changing the target single guide RNA (sgRNA) sequences, unlike ZFNs and TALENs that use protein guided DNA cleavage systems and can target only one gene at a time (Gaj et al. 2013).

The presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) sequence was first discovered in 1987 by Yoshizumi Ishino while working on DNA ligase of *Escherichia coli* (Ishino et al. 1987). However, the experimental evidence of its function as an adaptive immune system against viruses and phages was not discovered until 2007 by Rodolphe Barrangou (Barrangou et al. 2007). This discovery led to the first biotechnological breakthrough, that naturally occurring CRISPR/Cas systems could be used for immunization against phages (Barrangou & Horvath 2012). In 2008, the DNA targeting ability of CRISPR/Cas of *Staphylococcus epidermidis* was reported (Marraffini & Sontheimer 2008), providing evidence that this system can be found in different bacterial species. This gave way to discover different types of Cas enzymes with varying targeting abilities to widen the horizons of potential applications using CRISPR/Cas as a gene editing tool. The guiding ability of Cas9 by mature crRNA (CRISPR RNA) in *E. coli* against virus proliferation was identified in 2012 (Jinek et al. 2012). This discovery opened the gateway for the potential use of CRISPR/Cas9 for gene targeting and genome editing applications. The first report on the use of the CRISPR/Cas9 system for genome editing of fungi was established in 2013 on *Saccharomyces cerevisiae* (DiCarlo et al. 2013). Subsequently, stable CRISPR/Cas9 systems were established on *Trichoderma reesei* (Liu et al. 2015) and in several *Aspergillus* species (Nødvig et al. 2015). Since then, the CRISPR/Cas9 system has been successfully implemented for the manipulation of many fungal genomes, and many similar projects are underway for different species of fungi.

Here, we review the molecular mechanism of the CRISPR/Cas9 system, discuss phytopathogenic fungal genomes edited using the CRISPR/Cas9 system and its applications, and finally consider the challenges in manipulating phytopathogenic fungal genomes. The objective of this mini review is to provide a summarized overview of all up to date information and experiments related to the use of CRISPR/Cas9 for genome editing in plant pathogenic fungi and to provide a one stop information compilation for anyone interested in at a glance understanding of this robust molecular tool.

The native CRISPR/Cas system- The prokaryotic immune system

The CRISPR/Cas9 system works as an acquired immune system in prokaryotes by acting against any invasive foreign genetic elements from viruses or phages. Cas (CRISPR associated) genes coding for polymerases, nucleases, and helicases are an integral part of the bacterial adaptive immune system (Rath et al. 2015). The CRISPR locus is made of a conserved nucleotide sequence that is periodically repeated throughout the prokaryotic genome. During the initial invasion by the virus or phage, a small nucleotide sequence of the virus is processed by the nucleases of the CRISPR system, and these short nucleotide sequences are integrated to the CRISPR locus as spacers that are positioned between two repeats of the conserved sequence. The spacers act as transcriptional templates and create crRNA. This chimeric CRISPR array interacts with an auxiliary *trans* activating CRISPR RNA (tracrRNA) (Deltcheva et al. 2011, Chylinski et al. 2013), forming a duplex RNA known as guide RNA (gRNA) that in turn guides the Cas nuclease to cleave the genome of invading viruses or phages. Another small DNA sequence known as the Protospacer Adjacent Motif (PAM), present within the target DNA sequence, facilitates the specific targeting of the Cas nucleases. The PAM sequence is a short DNA sequence about three to five nucleotides in length. The presence of the PAM sequence is a strict requirement for Cas mediated nucleotide cleavage (Karvelis et al. 2015). Cas nucleases contain two domains, RuvC and HNH that cut the PAM containing strand and its complementary strand, respectively, to produce a double stranded break (DSB) (Chen et al. 2014) in the DNA of the invading bacteriophages or plasmids (Fig. 1). The presence of both domains in the Cas9 enzyme is important for a double stranded break.

CRISPR/Cas system can be classified into two major classes based on the components and the mechanism of action (Makarova et al. 2018). The class one system (type I, III, and IV) requires several complex effector proteins for functioning while in the class two system (type II and putative types V and VI); only one RNA guided nuclease is required to cleave the genetic material of the invading pathogen (Chylinski et al. 2014). Through the observations made on the mechanism of the native CRISPR/Cas9, researchers working on different aspects of this system collectively contributed to producing a feasible genome editing tool from the different components (Lander 2016).

Repurposing of the CRISPR/Cas9 system for genome engineering

Genome manipulation of higher eukaryotic organisms such as mammals, plants, and fungi is reported to be considerably difficult compared to other simpler organisms (Doench et al. 2014). The main reason for this is that many eukaryotic organisms such as filamentous fungi contain complex genomes that are diploid or polypoid (Wang & Coleman 2019). The edited genomes of these higher eukaryotes in many instances can produce reduced expression of the edited gene rather than producing a complete knockout of the function. Additionally, the transformation of higher eukaryotic cells is considerably more difficult compared with that of prokaryotic organisms due to their complex cellular organization. With the understanding of the biological function and the mechanism of the CRISPR/Cas9 system, the ability to modify the Cas9 to target specific nucleotide sequences in the host paved the way to developing a new method to solve this problem (Sander & Joung 2014). As a genetic tool, the CRISPR/Cas9 system could introduce heritable changes into the genome via precision insertions and deletions (Wu et al. 2014a, b). This technique has revolutionized the genome editing field; since most of the previous tools did not have the ability to cause stable and heritable changes to the target genomes.

The CRISPR/Cas9 system has been adopted for use in a wide range of eukaryotic organisms including yeast (DiCarlo et al. 2013), plants (Belhaj et al. 2013, Pandey et al. 2019), mammalian cells (Jinek et al. 2013, Cong et al. 2013, Manna et al. 2019) and fish (Hwang et al. 2013).

The aforementioned Class two system only requires a single Cas protein known as Cas9 (Type II) or closely related Cas12a; formerly Cpf1 (Type V) to induce double stranded breaks (Jinek et al. 2013). Thus, it is much easier to be converted into a genome editing tool. The combination of the tracrRNA with crRNA into a simple guide RNA (gRNA) facilitates the precise identification of the target site by the Cas9 enzyme. This feature is also unique to the type II system

(Cong et al. 2013, Hwang et al. 2013). Furthermore, Cas12a has the explicit ability to catalyze and process the pre-crRNA via innate ribonuclease activity, without requiring tracrRNA and RNase III (Safari et al. 2019). All these factors cumulatively make the type II and type V systems much simpler, more efficient, and more suitable for genome editing (Belhaj et al. 2013). In many stably transformed plant species, whole genome sequencing was performed to detect any off-target mutations caused by Cas9 or Cas12a. Only a low level of off-target mutations could be detected; giving testament to the high specificity of the nucleases (Chen et al. 2019).

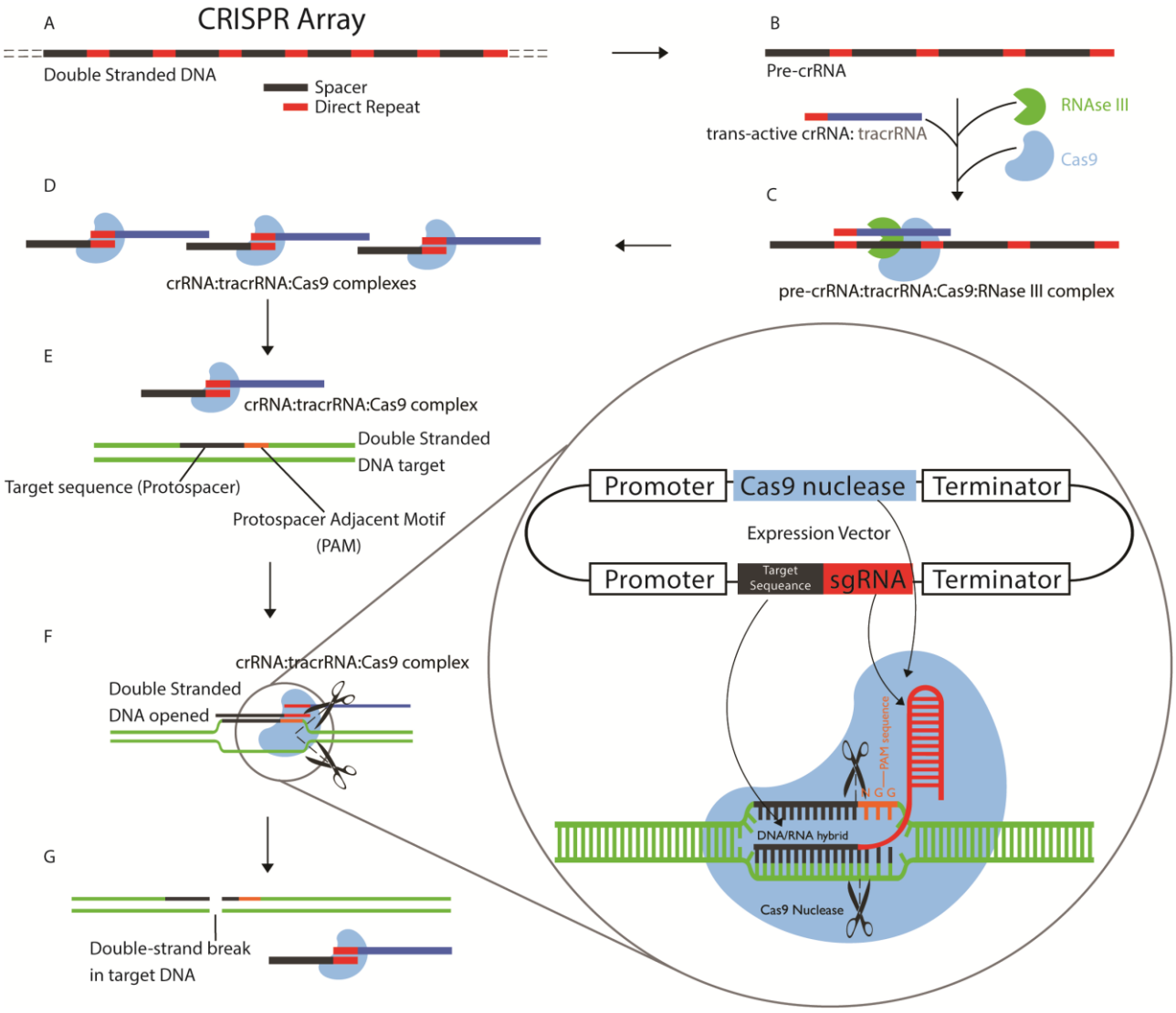

Fig. 1 – Outline of the native Type II bacterial CRISPR system and a blowup of CRISPR/Cas9 function during genome editing. A Natural CRISPR array is transcribed into pre-CRISPR RNA with the spacers and direct repeats. B The processing of pre-crRNA to crRNAs by tracrRNA and Cas9 and RNase III recruited to the tracrRNA. C Cleavage of the pre-crRNA. D forming a mature crRNA:tracrRNA:Cas9 complex. E crRNA:tracrRNA:Cas9 complex guided by the specific sequence of crRNA attaches to the target sequence. F cleavage of the double stranded DNA. During genome editing, sgRNA (single guide RNA) combination of both crRNA and tracrRNA works as the targeting sequence. G leaving a double stranded break at the target site.

The most significant change when converting naturally occurring CRISPR/Cas9 type II system into a genome editing tool is the fusion of crRNA and tracrRNA into one single RNA strand known as gRNA (guide RNA) or sgRNA (single guide RNA). This provides both the targeting specificity and the scaffolding/ binding ability to Cas9 (Doench et al. 2014). The Cas9:gRNA

complex recognizes the PAM sequence and creates a DSB at a specific target site to be repaired using the intrinsic cellular repair mechanisms of the host. The repair mechanism could either be non-homologous end joining (NHEJ) or homology directed repair (HDR) (Jasin & Haber 2016). The NHEJ mechanism is error prone, which leads to nearly random insertion and deletion mutations (i.e., indels) within the target sequence causing gene knockouts due to dysfunctional open reading frames (ORF), or by mutating a critical region of the protein for which the gene previously encoded. The other repair method, HDR, utilizes homologous recombination guided by a donor DNA template in repairing the break. This leads to precise gene replacement or knock in, mutagenesis, and gene corrections. Other genome editing systems such as ZFN and TALENS also use HDR as the repair mechanism for their genome edits. However, since the CRISPR/Cas9 system uses RNA to specify the editing location, this genome editing system is comparatively less expensive, less time consuming, and much more precise and scalable.

The *Streptococcus pyogenes* Cas9 (SpCas9) is the commonly used nuclease in the type II CRISPR/Cas system. SpCas9 accepts very frequently occurring NGG sequences as the PAM sequence, and thus a wide range of genes can be targeted by this enzyme. The nucleotide arrangement of the PAM sequence can vary depending on the origin of the Cas protein (Table 1) (Sander & Joung 2014).

Table 1 Different origins of Cas9 and their corresponding PAM sequences.

Cas9 species	**PAM sequence (5′-3′)**
Streptococcus pyogenes (Sp)	NGG or NAG
Staphylococcus aureus (Sa)	NGRRT or NGRRN
Neisseria meningitidis (NM)	NNNNGATT
Streptococcus thermophilus (St)	NNAGAAW
Treponema denticola (Td)	NAAAAC

The presence of both RuvC and HNH domains in the Cas9 is very important, as both domains are required to induce a DSB (Chen et al. 2014). If either one of the domains is mutated, Cas9 retains the ability to bind sgRNA but can only create a single stranded break or a nick. According to the nature of the edit to the genome, Cas9 with a mutated RuvC or HNH domain can be used as Cas9 nickase (Cas9n). When both the domains are mutated (dCas9), it can only bind sgRNA and does not possess the ability to cause double or single stranded breaks. The dCas9 has been used to instigate targeted gene expression without introducing permanent mutation to the gene (Moradpour & Abdulah 2020). There are several instances where Cas9n and dCas9 have been used for genome editing in mammalian cells (Gao et al. 2016, Hess et al. 2016). In fungi, dCas9 has been used in *Saccharomyces cerevisiae* (Gilbert et al. 2013) and Cas9n has been used in *Aspergillus niger* (Huang et al. 2019) for genome manipulation, but they have not been widely applied for gene editing in other fungal species.

Due to their immense importance in agriculture, phytopathogenic fungi are subjected to in depth molecular studies to identify genes involved in pathogenicity and potential drug target sites to develop efficient control measures. As previously mentioned, establishing a stable genome edit in filamentous fungi can be comparatively tedious. Difficulties in delivering gene editing components through the fungal cell wall, due to the presence of multinucleated cells and scarcity of suitable promoters and plasmids in the target fungi are some major factors contributing to this issue (Donohoue et al. 2018). The CRISPR/Cas9 system does not overcome all these problems but the editing efficiency is comparatively much higher than any conventional method used to edit the same fungal species previously (Nødvig et al. 2015).

Studying the genomes of plant pathogens to understand disease progression, possible control measures, and identification of genes related to the pathogenicity of the fungi has been attempted by many molecular biologists, with varying degrees of success. Since the dawn of the CRISPR/Cas9 system as a potential genome editing tool, many researchers have begun testing and using this system to edit phytopathogenic fungal genomes.

CRISPR/Cas9 for Phytopathogenic Fungi

The virulence of fungi is always attributed to several genes working together. Understanding how these genes work towards pathogenesis can be done via insertion, deletion, or replacement of the target genes. Not only does this allow the understanding of the function of a specific gene during pathogenesis, but also establishes target sites that could be later used for pathogen prevention purposes.

Due to the increased availability of whole genome sequences of fungi (Galagan 2005), the need for efficient genetic tools to exploit and use this information has become highly relevant. Precision genome editing by CRISPR/Cas9 has become an important tool to fill this void (Knott & Doudna 2018). The last few years have shown great potential for implementing the CRISPR/Cas9 system for genome editing in filamentous fungi. The CRISPR/Cas9 genome editing tool has been successfully used in model fungi such as *Neurospora crassa* (Matsu-ura et al. 2015), several *Aspergillus* species (Nødvig et al. 2015, 2018) including *Aspergillus aculeatus, A. brasiliensis, A. carbonarius, A. fumigatus, A. luchunesis , A.nidulans, A. niger, A. oryzae* (Fuller et al. 2015, Zhang et al. 2016, Katayama et al. 2016, Leynaud-Kieffer et al. 2019), *Myceliophthora thermophila* (Liu et al. 2017), *Penicillium chrysogenum* (Pohl et al. 2016), *Nodulisporium* sp. and *Sporormiella minima* (Zheng et al. 2017), *Talaromyces atroroseus* (Nielsen et al. 2017), *Trichoderma reesei* (Liu et al. 2015) demonstrating that the CRISPR/Cas9 system can be applied to a wide variety of filamentous fungi.

Establishing the CRISPR/Cas9 genome editing system has shown positive outcomes in many phytopathogenic fungi (Table 2). The CRISPR/Cas9 system was successful in disrupting genes within phytopathogenic fungi, in which targeted gene disruption was not possible before (Idnurm et al. 2017). Since the CRISPR/Cas9 system is comparatively more efficient and accurate, stable gene edits have been established in phytopathogenic fungi such as *Alternaria alternata* (Wenderoth et al. 2017), *Fusarium graminearum* (Gardiner & Kazan 2018), *F. fujikuroi* (Shi et al. 2019), *F. oxysporum* (Wang et al. 2018), *Leptosphaeria maculans* (Idnurm et al. 2017, Darma et al. 2019), *Phytophthora capsici* (Miao et al. 2018), *P. palmivora* (Gumtow et al. 2018), *P. sojae* (Fang & Tyler 2016), *Pyricularia oryzae* (Arazoe et al. 2015a, Foster et al. 2018, Yamato et al. 2019), *Sclerotinia sclerotiorum* (Li et al. 2018), *Shiaria bambusicola* (Deng et al. 2017), *Sporisorium scitamineum* (Lu et al. 2017), *Ustilago maydis* (Schuster et al. 2016) and *Ustilaginoidea virens* (Liang et al. 2018).

Many CRISPR related studies conducted on phytopathogenic fungi were performed in order to establish the first proof of principle of the functionality of the system within the target fungi (Schuster & Kahmann 2019). In many cases, genes that are not directly related to pathogenicity but that would provide a clear phenotypic change in the mutated state were used as a target to create sgRNA (Wenderoth et al. 2017). Once the system's feasibility is established in this manner, it can be modified to be used on any target gene within that particular organism.

The targeted DSB induced when implementing CRISPR/Cas9 for genome editing is one major contributing factor for the system's higher efficiency. In fungal species where the CRISPR/Cas9 system has been established, when the same donor DNA was introduced without the DSB, the efficiency of HDR was found to be comparatively reduced (Schuster & Kahmann 2019). In *P. chrysogenum,* when the CRISPR/Cas9 was used, the number of transformants with the desired mutation substantially increased in comparison to traditional HDR (Pohl et al. 2016).

In order to establish a successful CRISPR/Cas9 system, the expression of the sgRNA and the Cas9 should be carefully instigated, as they are the crucial working components of the system. The sgRNA expression should be activated under an efficient strong promoter. Optimized U6 promoters with higher transcriptional efficiency are used for the expression of sgRNA in many filamentous fungi (Schuster & Kahmann 2019). For example, in *P. oryzae,* sgRNA expressed under the U6 promoter was shown to have a better genome editing efficiency than that expressed under the TrpC promoter (Arazoe et al. 2015a). Almost all the phytopathogenic fungi edited using the CRISPR/Cas9 system have used the U6 promoter for the transcription of the sgRNA due to its high efficiency. The bacteriophage T7 promoter is also a better alternative when the U6 promoter is not

available or when its use is not practical (Fuller et al. 2015). The above-mentioned promoters are some of the frequently used promoters for genome editing in filamentous fungi. Since filamentous fungi possess a plethora of tRNA genes, identifying a more suitable promoter for the specific gene editing purposes should be considered. *In vitro* synthesis of the sgRNA is the more suitable choice if an efficient promoter could not be established for the expression of sgRNA within the target host. This would help in avoiding any error prone sgRNA being synthesized (Pohl et al. 2016).

The other crucial component of the CRISPR/Cas9 system, Cas9 endonuclease expression in filamentous fungi can be improved through codon optimization. Since Cas9 is of prokaryotic origin, efficient transcription of the endonuclease has been achieved for phytopathogenic fungi through human codon optimized Cas9 in *L. maculans* (Idnurm et al. 2017), *P. sojae* (Fang & Tyler 2016), *P. palmivora* (Gumtow et al. 2018) and *S. bambusicola* (Deng et al. 2017). Furthermore, the fungal codon optimized Cas9 used in Nødvig et al. (2015) was subsequently used for CRISPR/Cas9 system in many fungal species including the phytopathogen *A. alternata* (Wenderoth et al. 2017) and CRISPR/Cas9 system established in many phytopathogenic fungi has used Cas9 codon optimized for the expression in the host organism (Arazoe et al. 2015a, Foster et al. 2018, Shi et al. 2019). The Cas9 codon optimized for *P. oryzae* used in Arazoe et al. (2015) was subsequently used in *S. sclerotiorum* (Li et al. 2018) and *U. virens* (Liang et al. 2018). The common nuclear localization sequence (NLS) SV40 is also added to both ends of the Cas9 gene for accurate expression (Song et al. 2019). In order to facilitate the efficient expression of the Cas9 enzyme, placing the gene under a strong promoter is important. But, continuous expression of the Cas9 gene would lead to off-target effects and cellular stress. Hence, rather than using constitutive promoters, using an inducible promoter has been implemented as the smart alternative. For example, the promoter of the heat shock protein (hsp70) used for the expression of Cas9 in *U. maydis* was inducible by temperature changes (Schuster et al. 2016).

The expression of the Cas9 protein could either be done within the cell during transformation or can be added as preassembled RNPs (ribonucleoproteins) (Foster et al. 2018). The use of RNPs can be useful when implementing the CRISPR/Cas9 system across various genetic backgrounds and not only in specific strains engineered to express Cas9 or sgRNA that depend on DNA based expression cassettes for delivery (Al Abdallah et al. 2017).

The significance of the CRISPR/Cas9 mediated genome editing in phytopathogenic fungi

In the process of management of pathogenic fungi infecting economically important crops, plant disease resistant genes play a very important role. The discovery and identification of these plant genes can be challenging, thus characterizing the effector/avirulence molecules identified by these genes was found to be comparatively simpler (de Jonge et al. 2011). Through targeting these effector genes of phytopathogens using the CRISPR/Cas9 system, the mutated fungi can be used to trigger defense responses to guide the breeding of resistant genes which could potentially be an integral part of developing suitable disease management strategies.

Naturally occurring pathogen populations have the ability to overcome the disease resistance of crops grown in monoculture due to the absence of diversity in resistance against the pathogen (Van de Wouw & Idnurm 2019). The establishment of new fungal genotypes through targeting conserved, and therefore potentially essential, effectors could lead to the possibility of producing avirulent competitors for the plant pathogenic fungi in the field while also priming the plants against the virulent strains of the fungal pathogen. This could also lead to the identification of the corresponding resistance genes against these phytopathogens, which in turn could provide components in developing more durable resistance in the crops (Vleeshouwers & Oliver 2014).

For example, the rice blast disease caused by *P. oryzae* is considered as the most devastating rice disease in the world. Even though the disease epidemiology is quite well understood (Kim 2001, Greer & Webster 2001), preventive measures for this disease are still not totally effective. Implementing genome editing to undercut the virulence of this fungus could be potentially very useful. Gene editing using the CRISPR/Cas9 system has proven to be successful in *P. oryzae* in several studies (Arazoe et al. 2015a, Foster et al. 2018, Yamato et al. 2019). If this established

system could be used to produce avirulent strains by mutating or completely knocking out important pathogenicity related genes of the native wild type, the mutants could be used as a potential control measure by priming the crop against any aggressive virulent strains of the fungi.

Table 2 Phytopathogenic fungi and oomycetes edited using the CRISPR/Cas9 system

Species	**Disease**	**Modified gene(s)**	**Phenotype of the mutant**	**Significance of the edit**	**Mutation efficiency %**	**Reference(s)**
Alternaria alternata	Opportunistic plant pathogen causing variety of diseases in a wide host range.	Polyketide-synthase (pksA)	Loss of melanin deposition	Distinguishable phenotypic change in the mutant	-	Wenderoth et al. (2017)
		1,3,8-THN reductase encoding (brm2)	Loss of melanin deposition	Distinguishable phenotypic change in the mutant		
		Orotidine 5´-phosphate decarboxylase	Uracil auxotrophy	Efficient selection of the mutant		
		FphA LreA HogA	Reduced conidiation than the wildtype in the dark (reduced by 86% in FphA mutants, 51% in LreA mutants, 48% in HogA mutants) Germination of conidia was delayed in red, blue, green, and far-red light	Demonstrates that germination, sporulation, and secondary metabolism are light regulated in *A. alternata*	-	Igbalajobi et al. (2019)
Botrytis cinerea	Botrytis bunch rot in grapes Grey mold in many crops	Bos1 gene	Mutants are resistant against the fungicides iprodione (Ipr) and fludioxonil (Fld)	Efficient selection of the mutant	-	Leisen et al. (2020)

Table 2 Continued.

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
		botrydial (bot2) and botcinic acid (boa6) genes coding for key enzymes important for phytotoxins biosynthesis	-	Reduced pathogenicity in double mutants	-	
Colletotrichum sansevieriae	Anthracnose disease only in plants of the genus *Sansevieria*	Scytalone dehydratase gene (*SCD1*)	loss of melanin deposition	Distinguishable phenotypic change in the mutant (Pink colonies)	97.1	Nakamura et al. (2019)
Fusarium fujikuroi	"Foolish seedling" disease in rice	Fusarium cyclin C1 (*fcc1*)	Non-accumulation of a specific purple pigment	Distinguishable phenotypic change in the mutant	79.2	Shi et al. (2019)
		Orotidine-5'-phosphate decarboxylase (*ura3*)	Uracil auxotrophy	Efficient selection of the mutant		
		4'-phosphopantetheinyl transferase (*ppt1*)	Lysine auxotrophy	Efficient selection of the mutant		
Fusarium graminearum	Causal organism for wide range of diseases in cereal grain	Osmosensor histidine kinase 1 (FgOs1)	Resistance to the fungicide Fludioxonil	Simple phenotypic assay for mutant selection	1-10	Gardiner & Kazan (2018)

Table 2 Continued.

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
Fusarium oxysporum	Able to cause damage to tomato, banana, legumes cotton.	BIK1 gene encoding a putative polyketide synthase involved in the biosynthesis of bikaverin (Red pigment)	Inability to synthesize bikaverin	Distinguishable phenotypic change in the mutant	~50	Wang et al. (2018)
		URA5 -orotate phosphoribosyltransferase involved in pyrimidine biosynthesis	Uracil auxotrophy	Efficient selection of the mutant		
Leptosphaeria maculans	Blackleg disease on *Brassica* crops	osmosensing histidine kinase (hos1) gene	Loss of resistance to the fungicide iprodione Reduced growth under high salt conditions	Efficient selection of the mutant	-	Idnurm et al. (2017)
		polyketide synthase gene (*pks5*) abscisic acid-like 7 gene (*abl7*)	Changes in abscisic acid production	Pks1 and Abl1 genes are not involved in the pathogenicity of *Leptosphaeria maculans*		Darma et al. (2019)
Phytophthora capsici	Blight and fruit rot of peppers and other important commercial crops	Oxysterol binding protein-related protein-1 (ORP1)	High levels of resistance to the fungicide oxathiapiprolin	Efficient selection of the mutant	-	Miao et al. (2018)
Phytophthora palmivora	Bud rot and fruit rot in a wide range of crops	Cystatin-like extracellular protease inhibitors (PpalEPICs)	Increased papain sensitivity of in vitro growth and reduced	Efficient selection of the mutant and reduced virulence on	-	Gumtow et al. (2018)

Table 2 Continued.

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
			pathogenicity during infection of papaya fruits	the target host		
Pyricularia oryzae	Rice blast disease	Scytalone dehydrogenase (SDH) gene	loss of melanin deposition	Distinguishable phenotypic change in the mutant	36.1–80.5	Arazoe et al. (2015a)
		Polyketide synthase encoding ALB1 and RSY1	Albino or orange-red colonies formed	Distinguishable phenotypic change in the mutant	-	Foster et al. (2018)
		Scytalone dehydrogenase (SDH) gene	loss of melanin deposition	Distinguishable phenotypic change in the mutant	20-43	Yamato et al. (2019)
Sclerotinia sclerotiorum	Cottony rot, Watery soft rot, Stem rot, Drop, crown rot and Blossom blight	Oxalate biosynthesis gene Ssoah1	Overproduction of compound appressoria, Decreased pigmentation on the surface of sclerotia, Diffuse pattern of sclerotium development in culture	Distinguishable phenotypic change in the mutant Reduced virulence across a wide range of hosts	38-45	Li et al. (2018)
		Polyketide synthase gene for melanin biosynthesis Sspks13	Lack of pigmentation in compound appressoria	Distinguishable phenotypic change in the mutant		
Shiaria bambusicola	Bamboo blight	Major facilitator superfamily (MFS) gene in the hypocrellin gene cluster	No production of hypocrellin	Attenuated virulence on bamboo leaves	-	Deng et al. (2017)
Sporisorium scitamineum	Sugarcane smut	Mating related Mfa2 gene	Inability to get into filamentous growth after mating with a sex-opposite strain	Efficient selection of the mutant	21.7-39.1	Lu et al. (2017)

Table 2 Continued.

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
Ustilago maydis	Corn smut	bW2 and bE1	Loss of filament formation in charcoal containing agar plates	Distinguishable phenotypic change in the mutant	50–90	Schuster et al. (2016, 2018)
Ustilaginoidea virens	False smut disease in rice	*USTA* ustiloxin and *UvSLT2* MAP kinase genes	Increased sensitivity to cell wall stresses but tolerance to hyperosmotic or oxidative stresses	Efficient selection of the mutant	-	Liang et al. (2018)

Furthermore, many secondary metabolites produced by fungi have been reported as common virulence factors involved in the pathogenicity process (Darma et al. 2019). Phytohormones such as abscisic acid (ABA) produced by phytopathogenic fungi can manipulate plant immunity to promote disease development. For example, in *P. oryzae*, the deletion of abscisic acid producing gene *ABA4* reduced pathogenicity in rice plants and demonstrated that ABA increases spore germination and appressorium formation (Spence et al. 2015). The CRISPR/Cas9 systems established for *Phytophthora palmivora, Phytophthora sojae, Sclerotinia sclerotiorum*, and *Shiaria bambusicola* used genes related to pathogenicity as the targets and were successful in creating less virulent mutant strains (Fang & Tyler 2016, Deng et al. 2017, Lu et al. 2017, Gumtow et al. 2018). Through the manipulation of these candidate virulence genes, a mechanistic understanding of pathogenicity could be achieved. The resulting mutant stains can be used to test hypotheses on how these genes are involved in pathogenicity (Li et al. 2018). However, none of the CRISPR/Cas9 phytopathogenic transformants have been tested in the field as potential disease control agents.

Fungal phytopathogens spontaneously developing resistance towards chemical fungicides is a major concern in crop production. The CRISPR/Cas9 system established for *L. maculans* (Idnurm et al. 2017) and *P. capsici* (Miao et al. 2018) disrupted genes that were characterized previously as genes inferring fungicide resistance in the mutated state. *L. maculans* mutants previously discovered resistant to iprodione were used for gene disruption and *P. capsici* mutants resistant to oxathiapiprolin were produced using the CRISPR/Cas9 system. Characterization of these genes can be used to understand and develop novel fungicides like oxysterol binding protein homolog inhibitor fungicides for the control of *Phytophthora* spp., (Miao et al. 2018).

The use of chemical fungicides and reluctance to incorporate transgenic crops into their diets are important concerns for consumers. Thus, developing more resilient disease control strategies is an important issue. Implementing the CRISPR/Cas9 system to identify fungal genes that are required for growth and viability or effectors that are essential for virulence can provide crucial information to develop inspired disease control strategies in the future. Spray induced gene silencing (SIGS) is one such approach in which double stranded RNAs (dsRNAs) that target essential

pathogen genes are sprayed onto plant surfaces, which leads to disease control (Wang & Jin 2017). These could be used as species specific 'fungicides' due to specific targeting of genes.

Enhancing the biocontrol aptitudes of well-known fungal antagonists using the CRISPR/Cas9 system could also be a potential control method (Muñoz et al. 2019). The genetically enhanced antagonists with efficient metabolic pathways that trigger the biosynthesis of secreted proteins and secondary compounds could act as a frontline of defense against the invading fungal phytopathogens (Syed Ab Rahman et al. 2018). Through the programmable silencing of genes contributing to the competing reactions, the genes involved in the production of antagonistic substances such as diffusible antibiotics, toxins, extracellular cell wall degrading enzymes, and other volatile organic compounds could be enhanced (Van de Wouw & Idnurm 2019).

Though implementing CRISPR/Cas9 system for fungal genome editing is still in its infancy, the simplicity and efficiency of this system have shown to be much more beneficial and user friendly than any other genome editing tool currently available. This is due to the easy adaptability when used across different species of fungi. With the insights gained from the already existing systems of CRISPR/Cas9 for phytopathogenic fungi, the question of how this gene editing system can be used for other fungal species can be readily answered.

Challenges in CRISPR/Cas9

Although there are considerable merits with regards to the efficiency and specificity of the CRISPR/Cas9 system, when practically implementing the system, some drawbacks are also present (Zhang et al. 2014). These problems also need to be addressed to increase the efficiency and to obtain the full potential of this system.

One major concern with the CRISPR/Cas9 system is the off-target mutations. When using SpCas9 for genome editing, the NGG sequence acts as the PAM region. Utilizing only NGG as the single acceptable PAM sequence might lead to off-target mutations in instances where the target sequence has high homology elsewhere in the genome (Lin et al. 2014). This can be overcome by using novel *S. pyogenes* Cas9 variants with different PAM sequences or by using Cas9 homologs derived from species other than *S. pyogenes* (Table 1). Adapting Cpf1 (Cas12a) instead of Cas9 can also help in avoiding off-target mutations as the PAM sequence for Cas12a (TTTV) can avoid Cas9 PAM, if it frequently occurs throughout the target genome (Ungerer & Pakrasi 2016). Recently, genome editing using Cpf1 has successfully been implemented in filamentous fungi (Vanegas et al. 2019). On the other hand, the CRISPR/Cas9 system specifically designed for transient expression (Nagy et al. 2017) and systems with the ability to remove the Cas9 gene after genome editing (Wang et al. 2016) have also been implemented to avoid off-target mutations. Furthermore, using RNPs as the delivery method for the CRISPR/Cas9 components can also help in reducing off-target mutations. For example, in one study on *A. fumigatus*, where RNPs were used as the Cas9 delivery system, no off-target mutations could be detected (Al Abdallah et al. 2017).

Through the designing of highly specific sgRNAs, the low level of off targeting by the Cas nucleases could be avoided. Databases and software tools such as CasOT (Xiao et al. 2014), sgRNAcas9 (Xiao et al. 2014), E-CRISP (Heigwer et al. 2014), CHOPCHOP (Montague et al. 2014, Labun et al. 2016), CRISPRdirect (Naito et al. 2015), CRISPRscan (Moreno-Mateos et al. 2015) and CRISPOR (Haeussler et al. 2016) have been developed for easy search for potential off-targets within the genome and for the optimized production of the components of the CRIPSR/Cas9 system. The continuous expression of the Cas9 gene could be toxic to the host cells. The solution for this is the transient expression of CRISPR machinery to limit the strain to the cells. In addition, careful design of the sgRNA and limiting the longevity of the Cas9 sgRNA complex in a cell could increase the editing specificity of the CRISPR/Cas9 system.

Given that the specific targeting activity of the Cas9 enzyme depends on the presence of the PAM sequence downstream of the target sequence (Jinek et al. 2012), genes without the PAM sequence could not be targeted and edited using the CRISPR/Cas9 system. One way to overcome this PAM dependence is by using Cas variants with frequently occurring PAM sequences. However, rationally engineered SpCas9 variants with relaxed PAM recognition have also been

developed to reduce the PAM dependency of the CRISPR/Cas9 system (Nishimasu et al. 2018, Ren et al. 2019).

Retaining the required specificity of the designed sgRNA is another challenge in the CRISPR/Cas9 system. Post transcriptional modifications by RNA polymerase II within the host cell make it difficult for the synthesis of sgRNA, as such modifications would hinder the specificity. Without the specificity of the sgRNA, the targeting of the correct gene cannot be achieved.

Through the understanding of the mechanism of action, despite the challenges this method presents, the CRISPR/Cas9 for genome editing is now used in many different scientific fields for a variety of different purposes because of its abundant potential and the new possibilities it presents in the field of genome editing. Filamentous fungi play a very significant role in a variety of contexts. They can be consumed as a food source in the form of mushrooms, they can be an important plant, human and animal pathogens and they are used to produce natural compounds with medicinal properties, pigments used in textile industries and enzymes that can be used in biodegradation, biofuel production and fermentation. The CRISPR/Cas9 system with reverse genetics approach has been used in several studies in order to obtain certain desirable traits or to increase the production efficiency of certain chemicals or enzymes. In one study, white button mushroom *Agaricus bisporus* was modified to resist browning, and since no foreign DNA was introduced through CRIPSR/Cas9 system, it is exempt from GMO regulations in the United States (Waltz 2016). In *Myceliophthora thermophila* the CRISPR/Cas9 system allowed simultaneous deletion of four genes involved in cellulose production pathway, in which the resulting strain produced three-fold more lignocellulose in comparison to the parental strain (Liu et al. 2017). Another example is that through the silencing of genes that hinder the production of desired metabolites, the metabolic flux can be redirected to favor the production of the chemical or enzyme of our choice to increase the production efficiency and yield (Donohoue et al. 2018). Many Aspergillus species like *A. aculeatus, A. brasiliensis, A. carbonarius, A. luchuensis, A. nidulans,* and *A. niger,* which are a source of and producer of enzymes, were few of the first filamentous fungal species edited using the CRISPR/Cas9 system (Nødvig et al. 2015).

Conclusion

It is very important to consider which genome editing tool is more suitable for providing the answer to the relevant biological question pertaining to a research interest. There are many options now available for performing loss of function of genes of phytopathogens related to disease progression. In the past few years, the CRISPR/Cas9 system for genome editing has been successfully implemented in many fields and has advanced greatly. The simplicity, high efficiency, low cost, and the versatility of this system show great potential. There are many successful examples providing evidence that the CRISPR/Cas9 system is one of the best tools in the genome editing arsenal for studying fungal genomes, and the system will be implemented in fungal genetic research for years to come. Functional genomics has played a very important role in understanding the pathogenicity mechanism of many phytopathogenic fungi. Although many CRISPR/Cas9 related studies done up to now have only been to provide proof of function within the selected target organisms, the results gained by these studies can be further developed in order to combat plant diseases caused by many pathogenic fungi. As mentioned above, the CRISPR/Cas9 system is able to produce heritable changes in the genomes of the target phytopathogens. By understanding the involvement of a particular gene in the progression of a fungal disease, further studies can be conducted to provide suitable prevention strategies to reduce the devastating effects of phytopathogens like *P. oryzae* through precise editing to silence the pathogenicity related genes understand virulence related characteristics. Moreover, with the ability of CRISPR/Cas9 system to produce simultaneous gene edits throughout multiple locations, several pathogenicity related genes could be silenced in one go.

The safety of food crops is a very important factor to be considered when genetics related control measures are utilized. Since the CRISPR/Cas9 system does not introduce any foreign genes

into the mix of the existing genes, CRISPR/Cas9 edited phytopathogens can be considered relatively safe compared with phytopathogens modified with other gene editing tools. It should also be considered that the release of an avirulent mutant strain in the field could produce highly virulent strains after mating with the wild type. Thus, before considering the introduction of a mutated strain as a potential control measure in the field, the target organism should be selected from its native environment to avoid any genetic differences that would potentially give rise to novel, highly virulent combinations. Thus, the potential should be carefully examined and explored before any genome modified strain is released into the field as a control measure.

Resequencing the whole genome of the fungal host edited via the CRISPR/Cas9 system could be used to understand issues such as off-target effects that have not been addressed properly. With finding solutions to overcome the pitfalls faced by this impressive new toolbox, it is highly likely that future researchers will benefit massively by overcoming the challenges faced by CRISPR/Cas9 gene editing today, and future research will improve the ability to elucidate gene function, thus advancing the field for the further growth of science and technology.

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