RNA-Guided CRISPR-Cas9 System for Removal of Microbial Pathogens

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Abstract

CRISPR-Cas9 technology has been cherished and well appreciated by the scientific community. The popularity of CRISPR-Cas9 technology is because it provides simple and efficient directions for genome engineering with feasible applications in a broad range of organisms. It stands to reason that the development of CRISPR-Cas9 is probably among the greatest revolution in the field of molecular biology, ever since the discovery of PCR. Genome engineering of microbes and other organisms may open up newer avenues to achieve a dynamic ecosystem. In this chapter, research on the use of CRISPR-Cas9 technology as an anti-phytopathogenic arsenal has been highlighted. Furthermore, the engineered organism developed using CRISPR-Cas9 technology has also been explained. Besides the applicative side, the background and molecular mechanisms of the CRISPR-Cas9 system have been mentioned and explained thoroughly.

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12.1 Introduction

The behavior of an organism is determined by the genetic materials stored within the cell. Just like changing the alphabets of the word(s), either disturbs, recasts, or changes, the sense or information that has to be conveyed through a given sentence, with the same notion, the permanent changes, or modifications of genetic instruction stored within the cell(s) alter the behavior of the subjected model. Genome engineering is one way that helps to achieve such "precise" manipulation. It can be explained as an approach which deliberately modifies or creates correction or deletion in the genome of a living organism, with an intention to fulfill a particular purpose (Carr and Church 2009). Few of these purposes may be to explore and learn the biology of the subjected model and to develop microbes which are capable of acting as biosensors or help in efficient bioremediation (Carr and Church 2009). In comparison to the classical chemical synthesis protocols, genetically engineered organisms have shown a profound capability to generate industrially and commercially important products in a cost-effective manner (Nielsen et al. 2014).

The use of chemicals, radioactive mutagens or transposon elements is not favored when experiments demand precise manipulation as such approaches create random mutations besides the desired alteration (Carroll 2017). Eventually, several tools have been devised and developed that help achieve the targeted genome manipulation, with each having its own pros and cons. For example, the lambda red-based recombination is a simple and straightforward tool to use, but it provides a low frequency of recombinants (Yu et al. 2000). However, the frequency can be slightly improved by using the strains already harboring certain endonuclease knockouts (Mosberg et al. 2012). Several other tools have also been developed which involves customizable nuclease that can help target the desired sequences. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are examples of such customizable DNA scissors. TALEN exhibits superiority over ZFN as it is easy to design, can recognize longer sequences, and results in less off-target activity (Gupta and Musunuru 2014). Even though TALENs show precedent properties, it does lag in certain terms as compared to ZFN, such as cDNAs corresponding to pairs of TALENs are hard to deliver and express in cells as they tend to be larger in size (Gupta and Musunuru 2014). However, a new nuclease has to be designed if a new sequence is to be manipulated via ZFN or TALEN, making them costly, time consuming, and tedious to use.

Inspired by molecular mechanisms involved in CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-associated protein 9)-mediated immune system found in bacteria, the Cas9-gRNA complex has been exploited as a precise, scalable, cost-effective, and user-friendly genome engineering tool (Gasiunas et al. 2012; Jinek et al. 2012; Cong et al. 2013; Hsu et al. 2014). Considering the abovementioned features, CRISPR-Cas9 is a "tool of choice" among researcher for achieving the precise genome editing in an explicit way (Wiles et al. 2015). Reports suggest that more than 9000 research papers have been indexed in PubMed as regards "CRISPR" or "Cas9" since 2012 (Adli 2018). Initial in vitro studies highlight the caliber of Cas9 endonuclease for genome engineering purpose (Jinek et al. 2012). Over time, the use of Cas9 for genome editing has been explored in

human and mouse cell lines, along with a considerable number of a unicellular and multicellular organism, such as bacteria, yeast, mosquitos, zebrafish, and mice (Cong et al. 2013; DiCarlo et al. 2013; Gantz et al. 2015; Hisano et al. 2015; Jakočiūnas et al. 2015; Altenbuchner 2016; Singh et al. 2017; Burgio 2018). Diverse services are available that Cas9 or its recombinant form can provide for engineering purpose, some of which are listed as follows (Mali et al. 2013; Guo et al. 2016; Singh et al. 2018):

– Simplex or multiplex genome editing including double-strand breaks and nicks
– Efficient gene recombination
– Modulating genome architecture
– Gene(s) up- and downregulation

The significant increase of the world population calls for an escalating demand for food and shelter (Umesha et al. 2017). Therefore, to overcome the mentioned challenge, implementation of sustainable agricultural practices is required. A way to tackle the menace of food insecurity is to engineer microbes (Umesha et al. 2017). Analyzing the basic principle and mechanism of how microbes interact and behave in a given environment could possibly help to engineer microbes having the desired characters. The resultant engineered microbe would be meant for enhancing plant growth, for example, by increasing nutrient availability or to eradicate soil-borne diseases (Umesha et al. 2017). Eventually, several studies have been conducted which include the incorporation of CRISPR-Cas9-based systems for the evolution of engineered microbial tools that could possibly help to headway in achieving a sustainable agroecosystem.

In this chapter, we explore and explain how CRISPR-Cas9 technology can be used to eradicate several pathogenic microorganisms. By referring to such examples, the reader would be able to develop a better understanding about the use of CRISPR-Cas9 technology for dismissal of the pathogenic organism and subsequently be inspired to use such tactics for the development of a sustainable ecosystem. The chapter is divided into two sections. First, we explain the molecular mechanisms of the naturally occurring CRISPR-Cas9 system and the general working principle of CRISPR-Cas9 system. Second, we elaborate the applicative part of CRISPR-Cas9-based tools/systems in a range of microorganisms and parasites.

12.2 Background of CRISPR-Cas System

For an infection to strike and develop, phage has to go through several barriers that are imposed by the bacterial cell which help the bacteria to resist the infection (Shabbir et al. 2016). These barriers are broadly divided into innate and adaptive immune system. Tactics such as prevention of phage absorption or entry of its genetic material inside the cells are sensed or recognized by the bacterial innate immune system (Bikard and Marraffini 2012). In addition, digestion of the phage DNA by restriction endonuclease and aborting the infection by cell suicide are also examples of separate events performed as an inherent component of the innate immune system. The latter

mentioned strategy, named abortive infection, helps to protect the bacterial population rather than individual cell itself (Bikard and Marraffini 2012).

Another line of defense mechanism discovered in the bacterial cell is its adaptive immune system. CRISPR-Cas-mediated immunity is an example of such a defense mechanism against phage and other extrachromosomal elements. The CRISPR loci can be found in the archaeal and the bacterial genome that are marked as peculiar short repeats, spaced by short nucleotide (nt) sequence of encountered foreign DNA (Karginov and Hannon 2010). The "Cas" in "CRISPR-Cas" stands for CRISPR-associated sequence, and oftentimes, their product facilitates the CRISPR loci to demonstrate the adaptive nature of the immune system as well as helps to recognize and cleave the foreign sequence (either DNA or RNA depending on the type of CRISPR-Cas system).

The quest for CRISPR began in the year 1987 (Ishino et al. 1987), where some unique interspaced repeats were observed in the genome of *E. coli*. The term "clustered regularly interspaced short palindromic repeats" (CRISPR) was coined in 2002, and the possible biological role of CRISPR in the immune system was proposed in 2006 (Makarova et al. 2006; Karginov and Hannon 2010). However, in 2010 through in vivo studies, discovery as regards to the adaptive nature of CRISPR-Cas-mediated immune system was disclosed (Garneau et al. 2010). With the current knowledge, CRISPR-Cas systems are classified into class I and class II. Because of the frequent recombination and absence of universal Cas gene within the different CRISPR-Cas systems, classifying them becomes both tedious and difficult (Koonin et al. 2017). Currently, the classification is made by considering several factors including availability of different signature Cas genes and sequence similarities of shared Cas genes across different CRISPR-Cas systems. Moreover, the structure of CRISPR and organization of genes in the CRISPR loci are also taken into account while classifying CRISPR-Cas system (Koonin et al. 2017).

Class I consists of type I, III, and IV CRISPR-Cas system. Among all the types, types I and III are most commonly found, and contrary to this, type IV is a rare occurring CRISPR-Cas system. Generally, class I CRISPR-Cas system requires multiple subunits and proteins to demonstrate its defense mechanism, while class II CRISPR-Cas system does the same job by recruiting a single and simple multidomain protein rather than the involving multiple subunits and proteins. Furthermore, class II CRISPR-Cas system has very basic and uniform CRISPR-Cas loci as compared to class I system. CRISPR-Cas9-mediated immune system is an example of class II type II CRISPR-Cas system. In addition to type II, class II also includes type V and VI CRISPR-Cas system (Koonin et al. 2017). Research is on the progress about new discovery of CRISPR which is expanding every time. It can be further used for a variety of applications in environment, biofuels, agriculture, and more.

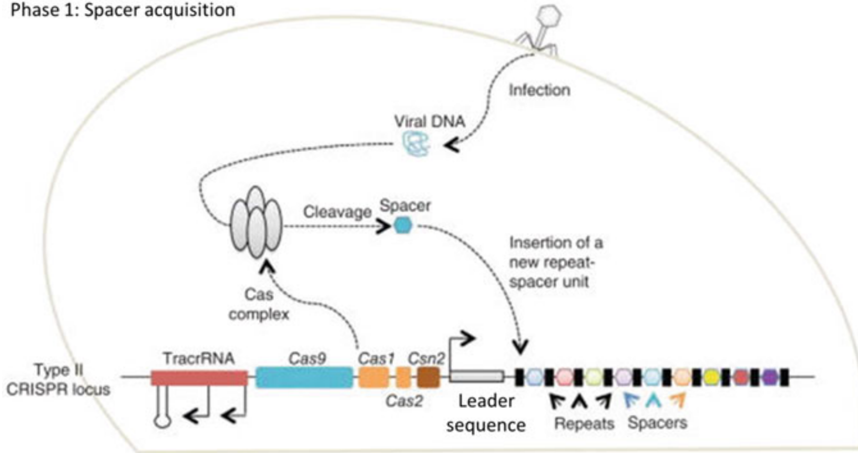
12.3 Molecular Mechanism of CRISPR-Cas9 System

CRISPR-Cas9-mediated immune system recruits a special kind of endonuclease that pursues for a target sequence (Rath et al. 2015). The hunt to cleave a particular sequence is guided by an RNA molecule which is called a guide RNA (gRNA). With

the involvement of a distinct kind of multi-domain endonuclease and gRNA, the CRISPR-Cas9 system is able to find a target sequence from the pool of genetic instructions present within the cell. On target recognition, the system activates itself and cleaves the recognized sequence (Rath et al. 2015). The target sequence is recognized based on the complementarity between the gRNA molecule and the DNA sequence. With that arises a question on how the cell gets to know as regards to the sequence of gRNA that would target a particular foreign sequence.

Being a part of the bacterial adaptive immune system, CRISPR-Cas9 has the ability to memorize the previously encountered sequence (Rath et al. 2015). Whenever a phage genome enters into the cell, the Cas proteins acquire a certain portion of foreign nucleotide sequences between two "repeat sequences" found within the CRISPR locus (Wei et al. 2015). Thereafter, the incorporated sequence would act as a memory for the host organism. The sequence of the phage genome selected to be linked within the CRISPR locus is known as protospacer. Once it gets integrated into the bacterial genome, it is termed as a spacer (Shah et al. 2013). However, the incorporation is complete in a way that the resultant product would lead to replication of the repeat sequence found adjacent to the leader sequence in the CRISPR locus. Furthermore, the incorporated spacer would be present between the newly duplicated repeat sequences (Wei et al. 2015). This scenario ensures the maintenance of the repeat-spacer architecture. The integration of 33–35-nt-long protospacer sequence is done by identification of the proximal end of the leader sequence and 5' end of its adjacent repeat sequence, through Cas1–Cas2 protein complex (Heler et al. 2015; Wei et al. 2015). The entire event of incorporating the protospacer sequence into the genome of the cell, specifically within CRISPR locus, is called spacer acquisition or more commonly adaption (Fig. 12.1).

However, any random sequence is not selected to act as a protospacer during the spacer acquisition. The sequence next to the protospacer adjacent motif (PAM) is selected and acts as a potential spacer sequence for CRISPR locus. PAM motif is specific short three- to five-nucleotide-long sequence that is recognized by a multidomain Cas9 nuclease (Mojica et al. 2009; Heler et al. 2015). Once recognized by Cas9, the Cas1–Cas2 protein complex would serve as an integrase and carry out steps necessary for spacer acquisition. Preference to a nonself protospacer over self seems to be hugely dependent on RecBCD-mediated DNA repair mechanism. Whenever there is stalled replication fork or DNA damage, RecBCD would cleave the dsDNA into short single-stranded DNA until it reaches the Chi site (Levy et al. 2015; Wang et al. 2015). Once it approaches Chi site, the 5' end has been degraded to some extent, producing 3' overhangs (Dillingham and Kowalczykowski 2008). The frequency of occurrence of Chi site would be less in the nonself DNA compared to the self DNA. Thus, during the RecBCD-mediated genome repair, the phage DNA could produce more degraded short single-stranded DNA in comparison to self DNA (Levy et al. 2015). It is assumed, through unknown mechanism, that the short ssDNAs are converted into dsDNA molecules. The potential PAM motif of protospacer is recognized by the Cas9 nuclease that recruits Cas1–Cas2 complex to integrate the 33–35 bp sequence (protospacer) into the CRISPR loci (Wright et al. 2016).



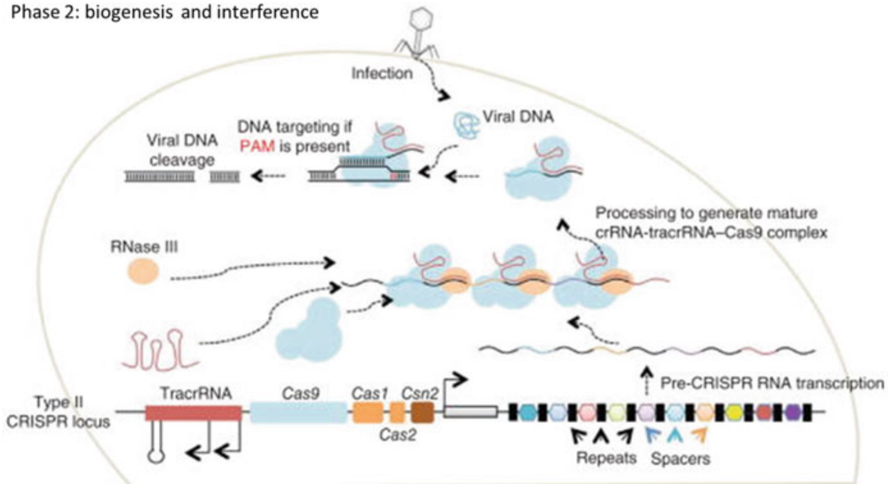


Fig. 12.1 CRISPR-Cas system defense mechanism by acquisition, RNA processing, and interference. Phase 1, on entry of viral DNA into the cell, the Cas1–Cas2 complex integrates the sequence adjacent to the PAM motif into the CRISPR loci. The first repeat unit and protospacer sequence is incorporated between the newly formed repeat sequence. Cas9 helps in the recognition of the PAM sequence. Cas1–Cas2 as a whole is shown as Cas complex. This step of protospacer incorporation is referred as spacer acquisition. In phase 2, the CRISPR loci are transcribed as a whole and are termed as pre-CRISPR RNA. With the formation of crRNA/tracrRNA-Cas9 complex and involvement of RNase III, the pre-crRNA is converted to mature CRISPR RNA, and this step is known as biogenesis. With the emergence of mature crRNA, Cas9 forms complex with it and hunts for the target sequence. The target sequence tends to lie next to the PAM motif, and it is complementary to the spacer sequence of mature crRNA. This step of DNA cleavage is called interference. (Figure reproduced with permission from Mali et al. 2013© (2013) Springer Nature)

The leader sequence of CRISPR locus not only helps in spacer acquisition but also helps to transcribe the CRISPR-RNA (crRNA). The leader sequence is present on the upstream of the repeat-spacer architecture. Furthermore, promoter sequence is embedded inside it (Carte et al. 2014). This sequence promotes the transcription of the so-called repeat-spacer architecture as a whole, and the resultant RNA obtained is called pre-crRNA. However, it is not the pre-crRNA but the mature crRNA (in applicative biology called as gRNA) that guides the multi-domain Cas9 nuclease to target the particular sequence (Brouns et al. 2008; Karvelis et al. 2013). RNase III and tracrRNA are responsible to convert the pre-crRNA into the mature crRNAs (Deltcheva et al. 2011; Garrett et al. 2015). A set of events that is performed following the transcription of pre-crRNA to its maturation is categorized as biogenesis. The sequence of tracrRNA complements the repeat sequence found within the pre-crRNA and is meant to form tracrRNA/pre-crRNA complex which is stabilized by the Cas9 nuclease (Garrett et al. 2015).

The transcribed repeat-spacer architecture has hydrogen-bonded tracrRNA on each and every repeat sequence of pre-crRNA. The gene corresponding to tracrRNA is located within the CRISPR-Cas loci (Chylinski et al. 2013; Chylinski et al. 2014). The role of RNase III in biogenesis is to cleave the tracrRNA:pre-crRNA complex at the repeat sequence, thus leading to the formation of several crRNAs (Deltcheva et al. 2011) (Fig. 12.1). The resultant crRNAs consist of a spacer and some flanking repeat sequences (on both ends of crRNA) which are attached to the nucleotide sequence of tracrRNA. The nucleotide sequences of repeats corresponding to the 5’ of the produced crRNAs as well as some nucleotide sequence of the spacer are trimmed by some unknown nuclease to form a mature crRNA (Wright et al. 2016).

On the binding of mature crRNA to the Cas9 nuclease, there is a conformational shift from its auto-inhibited state, thus allowing the Cas9 nuclease to look for the target sequence (Jinek et al. 2014). Furthermore, two arginine residues found in the PAM-interacting domain of the Cas9 would be pre-positioned to search for the PAM motif in dsDNA (Wright et al. 2016). On recognizing the PAM motif, the nuclease loses the first 10–12 nt sequences starting from the proximal end of PAM motif found in dsDNA (Szczelkun et al. 2014). The first 10–12 nt sequences from the 3’ end of the PAM motif is called the seed sequence (Szczelkun et al. 2014). Once the seed region and its distal end sequence have opened up, the mature crRNA forms complementarity with the seed over and above its distal end nucleotide sequence. Only a few, if not all, mismatches are tolerated in the seed region of the open DNA with the mature crRNA (Wright et al. 2016). Once a perfect or near-perfect match is achieved, the HNH domain of Cas9 nuclease is positioned in its catalytic active position. This, in turn, allosterically regulates and activates the RuvC domain of that particular nuclease (Sternberg et al. 2015). HNH and RuvC-like domain are meant to cleave target and the nontarget strand of dsDNA, respectively (Jinek et al. 2012). The entire process to hunt for the target sequence with the help of Cas9 nuclease is termed as interference (Fig. 12.1). Along with CRISPR-Cas9 system, the molecular mechanism of several other CRISPR-Cas systems is being studied, which promises not only effective genome engineering but also pathogen detection (Knott and Doudna 2018; Gootenberg et al. 2018; Khambhati et al. 2018).

12.4 The CRISPR-Cas9 Genome Editing Technology

Investigating the biological systems does satisfy one's curiosity, as it helps to give an answer to a particular question. The efforts put by researchers are highly appreciated as it helps unveil the behavior of the subjected system. However, if the knowledge gained by investigating the basic biology is somehow translated to some kind of applicative technology, then it would facilitate the upliftment of the human society. For example, increased understanding with regards to the behavior of restriction endonuclease has led to the development of several molecular cloning tools along with several crime investigating techniques. The developed molecular cloning tools have acted as a major support for biotechnology-based industries and also in exploring the basic biology (Roberts 2005).

Likewise, much knowledge has been gained about the working principle of the CRISPR-Cas9 system that has helped to develop an efficient and easy to use genome engineering tool(s). The in vitro proof-of-concept was demonstrated by Jinek and colleagues in 2012 (Jinek et al. 2012). The CRISPR-Cas9 can function as a tool for making precise double-strand breaks by expressing Cas9 protein inside a cell with the help of an expression vector. Simultaneously, gRNA can direct Cas9 towards the target sequence (Cong et al. 2013). The introduction of double-stranded break leads to the activation of repair mechanism by nonhomologous end joining (NHEJ) or by the homology-directed repair (HDR) pathway. The activation of such a mechanism facilitates the modification or replacement of the desired sequence or further can help to create gene knockouts (Singh et al. 2017; Singh et al. 2018).

Rather than expressing the tracrRNA and the spacer sequence individually, the single gRNA (sgRNA) can be chemically synthesized and expressed directly to cleave the desired sequence via Cas9. The sgRNA includes a tracrRNA and spacer sequence (12–13 nt target complementary sequence), which are physically linked as one with the help of a linker sequence. However, the only requirement is that the selected target sequence in dsDNA should be adjacent to the PAM motif (Jinek et al. 2012; Cong et al. 2013). A particular sequence of PAM motif corresponds to a particular kind of Cas9 protein, consequently, by identifying the different variants of Cas9 proteins and its corresponding PAM site (Singh et al. 2017) one can increase the proximity of using the tool to target the wide range of sequence (Fig. 12.2).

The popularity of Cas9 is not only confined to make precise double-strand breaks but also a mutant version named dead Cas9 (dCas9) is known for regulating the gene expression (La Russa and Qi 2015). The dCas9 is unable to produce double-strand breaks because of having mutation in the active domain of the protein (RuvC and HNH domains). As its applicative side, one can program the dCas9 with the help of gRNA molecule to recognize and anchor onto a particular promoter sequence. The promoter sequence of choice could be upstream to the gene sequence of which the downregulation is desired. The anchored dCas9 interferes with the RNA polymerase that is supposed to bind with the promoter sequence and prevents it from carrying out transcription. Furthermore, an ongoing transcription can be brought to a hold by targeting the gene sequence rather than the promoter sequence (Bikard et al. 2013). Thus, dCas9 can be used as a molecular tool to downregulate the gene expression. In a similar manner, it can also help in the upregulation of the desired gene by allowing

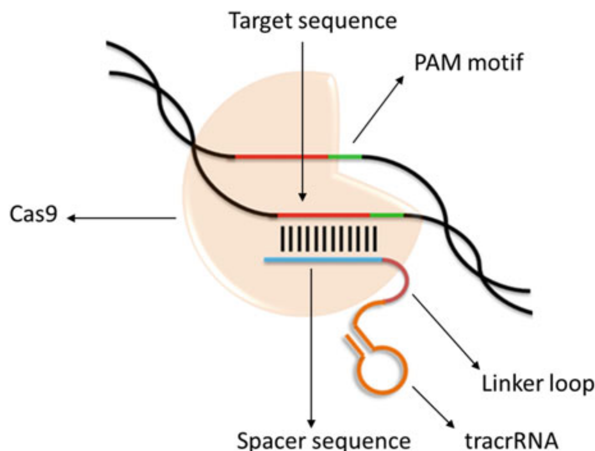


Fig. 12.2 Schematic representation of genome editing using CRISPR-Cas9 system. The guide RNA, which includes 20-nt spacer is linked to tracrRNA with the help of a linker sequence (forming a loop). The Cas9 nuclease in the presence of guide RNA forms a complex that can bind with the target region in the presence of PAM sequence that allows to create a double-strand breaks in order to generate a gene knockout. (Jinek et al. 2012)

the fused form of dCas9 that binds onto the promoter sequence. For example, the fusion of the trans-activator domain with dCas9 may allow us to activate the gene expression. The fused dCas9 could possibly have omega (ω) subunit of RNA polymerase (RNAP) linked to it, which allows the recruitment of RNAP to the promoter region. The recombinant form of the dCas9 protein would recruit the RNA polymerase onto the promoter sequence and activate the transcription of the desired gene (Bikard et al. 2013).

12.5 Potential Application of CRISPR-Cas9 System for Removal of Pathogens

12.5.1 Genome Editing of Fungi Using CRISPR-Cas9 System

Fungi are ubiquitous in nature that may either occur as a unicellular organism or as a highly complex multicellular organization. Depending upon their habitat, fungi delineate a range of infective properties. Being highly efficient decomposers, fungi feed on the dead and decomposed the matter, channelizing the important elements such as carbon, nitrogen, salts, and other organic matter back into the environment. Other than its involvement in human disease and infections, they are the general spoilers of food and crops. Fungi are also associated with synthesis of high value naturally available biologically active products in agro-based, food, and pharmaceutical industry. Parasitic fungi residing over plants and crops cause mildew and rust, resulting in huge monetary losses every year. As far as the higher organisms are concerned, the numbers of fungi involved in animal and plant diseases are relatively

less as compared to bacterial ones. Fungi are often characterized based on their structure or the fruiting bodies they form, their life cycle, and the type and arrangement of the spore (reproductive or distributional) they produce.

Predominantly, fungi are characterized into three major groups:

(i) Unicellular microscopic yeasts

Yeasts are single-celled, eukaryotic members of the fungus kingdom constituting about 1% of the total fungal population. They are small, round lemon-like cells sized ~5 μm in diameter (Duina et al. 2014). Yeasts multiply asexually by budding a daughter cell off from the parent cell (e.g., *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*).

(ii) Multicellular filamentous molds

Molds are composed of fine threadlike structures called hyphae. These hyphae divide repeatedly at the tip and form mesh-like arrangements by intertwining with neighboring hyphae. This mesh-like network is called mycelium. The digestive enzymes are present at the tips of the hyphae that digest the organic matter surrounding their habitat which is then utilized as the source of energy. Molds form spores on their aerial branches, which are nothing but their reproductive structures enclosed within a protective covering in order to protect them against harsh and unsuitable climatic condition or a state of starvation. The spores spread via wind, insects, or rain. When the conditions become favorable, the resistant spores germinate into a fresh new fungus and produce new hyphae (e.g., *Rhizopus nigricans* and *Spinellus fusiger*).

(iii) Macroscopic filamentous fungi bearing large fruiting bodies

Similar to filamentous molds, mushrooms also bear spores that are utilized to propagate and maintain their generations. However, a major difference that distinguishes mushrooms from molds is that mushrooms form visible fruiting bodies to hold the spores together. The fruiting body is commonly associated to a cap-like structure of mushroom, which is composed of densely packed hyphae, and the gills underneath the cap is where the spores reside (e.g., *Agaricus bisporus* (edible button mushrooms) and *Amanita phalloides* (deadly poisonous mushroom)).

The close associations of the filamentous fungi with humans have led to the thought of manipulation of the genome of higher fungi to extract high-value bulk and fine bioactive natural compounds (Thrane et al. 2007). Owing to their simple structure and genetic composition, yeast is used as model organism to study genetics. Yeasts *Saccharomyces* are extensively used in the baking and brewing industries. Considering their ability to synthesize large amounts of proteins along with the presence of a complex yet efficient post-translational processing system, a diverse range of fungus, namely, *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei*, are used in the synthesis of enzymes and valuable proteins, the species being regarded safe by the US Food and Drug Administration (Ward 2012; Shi et al. 2017).

Filamentous fungi find applications in the synthesis of antibiotics, organic compounds, novel drugs, pigments, and so on. Filamentous fungi are applied in the synthesis of a variety of pharmaceutical products. Few of the cholesterol-

lowering drugs are statins, such as lovastatin and mevastatin, produced from *Aspergillus terreus* and *Penicillium citinium*, respectively (Barrios-González and Miranda 2010). Antifungal medication such as griseofulvin that is used to treat infections of the skin, scalp, nails, and groin is developed from the *Tinea* strains of fungi, and echinocandins, popularly known as "penicillin of antifungals" (Kumar and Jha 2017), along with penicillins, cephalosporins (Hamad 2010), and a few other antibiotics of the β-lactam class (Cho et al. 2014) that are derived from filamentous fungi. Conversely, some fungi produce mycotoxins that are pernicious to humans and plants. Some opportunistic pathogenic forms of yeasts such as *Candida* cause infections in immunocompromised individuals. Toxins such as fumonisins, trichothecenes, and aflatoxins from *Fusarium verticillioides*, *Fusarium graminearum*, and *Aspergillus flavus*, respectively, are to name a few (Woloshuk and Shim 2013), that contaminate the food products or infect crops, ultimately leading to huge economic losses (Harris et al. 2016; Lecellier et al. 2015). Therefore, to subside the adverse effects generated by fungi and potentiate their use for creating valuable yet economic products, it is necessary to understand their genetic build-up and reconstruct them accordingly. A pressing need has arisen to edit the fungal genome in order to produce large amounts of complex and useful chemicals for industrial and agricultural applications. Figure 12.3 shows a quick look of CRISPR-Cas9-assisted genome editing in microbes and parasite.

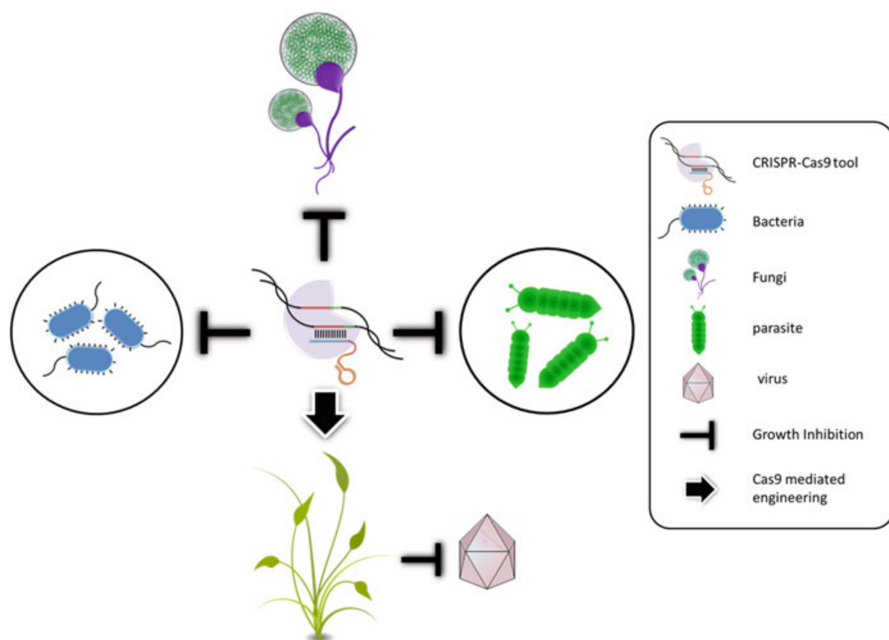


Fig. 12.3 Applications of CRISPR-Cas9 technology toward plant protection and better growth. It has demonstrated the inhibition of the plant pathogenic bacteria, fungi, and parasite. Plant has been modified using CRISPR-Cas9 system for viral resistance

The CRISPR-Cas9 platform offers to orchestrate the fungal genome and engineer its synthetic gene circuits accordingly. The platform thus finds application in decoding pathogenicity, synthesizing valuable metabolites and drugs, and boosting bioenergy processes (Deng et al. 2017). A key aspect of designing a highly efficient CRISPR-Cas9 tool is to have a coherent promoter. For the fungal system, often the poly III U6 promoter is used for expressing the sgRNA sequences, the promoter being excellent in transcription (Miyagishi and Taira 2002). In case of unavailability of the U6 promoter, *Saccharomyces cerevisiae* promoter SNR52 works fine in *Aspergillus fumigatus* (Fuller et al. 2015) and *Neurospora crassa* (Matsu-ura et al. 2015), or the T7 promoter of bacteriophage for activating sgRNA for *Trichoderma reesei* (Liu et al. 2015) and *Penicillium chrysogenum* (Pohl et al. 2016) has been described.

The desirable Cas9 expression can be attained by incorporating suitable inducible or constitutive promoters. Constitutive promoters that are used for the expression of Cas9 in filamentous fungi include gpdA (Zhang et al. 2016) and translation-elongation factor 1α (tef1) (Kuivanen et al. 2016; Liu et al. 2017). Codon optimization of Cas9 could readily enhance the genome editing ability in filamentous fungi, the proof of which has been observed in multiple strains including *Aspergillus* (Fuller et al. 2015; Katayama et al. 2016; Weber et al. 2016), β-lactam-producing *Penicillium chrysogenum* (formerly known as *Penicillium notatum*) (Pohl et al. 2016), *Trichoderma reesei* that is a rich source of industrially valuable cellulolytic enzyme (Liu et al. 2015), and corn smut causing *Ustilago maydis* strain (Schuster et al. 2016). Essentially, three main approaches are employed when it comes to transforming the Cas9-sgRNA complex into filamentous fungi which include poly-ethylene glycol (PEG) transformation, *Agrobacterium*-mediated transformation (AMT), and blastospore-mediated transformation (Deng et al. 2017). The PEG-$CaCl_2$ transformation is relatively simple, and the method makes use of protoplasts generated from hypha and germinated spores, whereas the *Agrobacterium* and the host are directly co-cultured for AMT-based transformations. Else ways, a blastospore transformation is facilitated by lithium acetate-mediated delivery of DNA.

The mechanics of the CRISPR-Cas9 system can be regulated in several ways. The inhibition of the system is delineated by a set of natural inhibitors, called anti-CRISPR (Acr) proteins. The proteins bind with Cas9 in the CRISPR-Cas9 complex, thereby functioning as its "off-switches" and thus get hold over the Cas9 activity. A bright side of the anti-CRISPR (Acr) proteins is their involvement in reducing the chances of CRISPR-Cas9 off-targeting and therefore diminishing the side effects (Shin et al. 2017). For example, a CRISPR inhibitory protein called AcrIIA4, which binds to the CRISPR-Cas9 complex involved in sgRNA-mediated targeting in sniping off the mutated hemoglobin gene responsible for sickle cell anemia, reduces the possibility of off-targeting by fourfold (Shin et al. 2017).

Light and chemical means have been used for corrections in the genome in *Aspergillus fumigatus* and many other eukaryotic strains (Zhang et al. 2016). Apart from this, it is also possible to maneuver genome editing by spatially and temporally regulating Cas9 activity through the anti-CRISPR system (Pawluk et al. 2016; Rauch et al. 2017). Majority of fungal infections occur as a function of the synergistic effect of multiple genes. CRISPR-Cas9 complex with utmost efficiency

can target the toxin-producing genes in most fungal pathogens. *Phytophthora sojae* is an oomycete (water mold) that attacks plants of agricultural and ornamental importance. The plant pathogen causes the stem and root in soybean plants to rot, leading to a whopping loss of soybean crops. Disrupting and replacing the *Avr4/6* gene belonging to the superfamily of RXLR virulence effector proteins using CRISPR-Cas9 enable a better control over the pathogen (Fang and Tyler 2016).

Attempts have also been taken to edit the genome of a commonly found saprophyte (an organism that feeds on dead and decaying organic matter) *A. fumigatus* (Zhang et al. 2016). Modifications such as disrupting the polyketide synthase gene (*pksP*), an important enzyme involved in toxin biosynthesis, hamper the ability of *A. fumigatus* to produce toxic compounds and consequently minimize the detrimental effects on the host (Fuller et al. 2015). Engineering entomopathogenic fungi *Beauveria bassiana*, a parasite that grows over arthropods and causes white muscardine disease, can serve the purpose of insect or pest management (Chen et al. 2017).

Cellulose is the most abundant biomass on earth and has proved to be a lucrative resource for the paper, wood, fiber, fodder, cosmetic, and pharmaceutical industries (Shokri and Adibkia 2013). The humongous quantity of agro-based waste can be utilized to generate a profitable product. The degradation of cellulose is catalyzed by an enzyme called cellulase. Some fungi belonging to the genus *Aspergillus*, *Rhizopus*, *Trichoderma*, *Fusarium*, *Neurospora*, and *Penicillium* can very easily degrade the cellulose into simple constituents (Sajith et al. 2016). Incorporating the CRISPR-Cas9 system to enhance the production of cellulase in several fungal strains has shown some assuring results in minimizing the size of the agro-waste generated. CRISPR-Cas9-mediated upregulation of transcriptional factor (clr-2) results in an increase of cellulases in *Neurospora crassa* (Matsu-ura et al. 2015).

Myceliophthora, an ascomycete (sac fungi), is a thermophilic fungus capable of hydrolyzing cellulose and hemicellulose and utilizes that as the energy source during the unavailability of proper carbon source, especially when the temperature rises and the soil becomes dry. It is possible to increase the production of cellulase by fivefold as compared to the parent strain just by disrupting 3–4 genes involved in the biosynthetic pathway (Liu et al. 2017). The CRISPR-Cas9 system is currently less explored in plant-beneficiary fungi, but most certainly that can be expanded to achieve better agricultural productivity and yields.

12.5.2 CRISPR-Cas9 as Antiviral Agent

Viruses are small particles that can infect all types of organisms. Oftentimes, it causes diseases and affects the health of plants and animals. According to the World Health Organization, HIV infects the life of about 35 million people with over 70 million cases of infection reported until 2017 (WHO 2018). Lifelong antiretroviral therapy (ART) may help manage the disease to some extent, but it cannot cure the disease completely. The virus delineates its infective properties by inserting its genes into the host genome which then continues to replicate latently. The main targets of the virus are the CD4+ cells, macrophages, and follicular dendritic cells. Restricting the spread of the infection is pretty challenging because the available antiviral

compounds fail to target the integrated proviral genome and the viruses are quick to rebound after ART cessation. Other than this, the viruses even tend to hide into tissue spaces of the central nervous system. Designing an RNA-guided CRISPR-Cas9 tool to target the regulatory genes of HIV-1 can be an effective solution. A lentiviral vector mode of transduction is a process where the gRNA cloned into lentiCRISPRv2 has been used to specifically target the regulatory genes *tat* and *rev* (Ophinni et al. 2018). Transduction of the tool into 293T and HeLa cell lines successfully eliminated the stably expressing Tat and Rev proteins. As a result, the functional assay of *tat* and *rev* genes revealed a significant reduction in the level of HIV-1 promoter-driven luciferase expression and inhibition of gp120 activity (Ophinni et al. 2018). Genome editing through CRISPR-Cas9 has also been used for herpes viruses (Chen et al. 2018) including the herpes simplex virus 1 and 2 (HSV-1 and HSV-2) (Johnson et al. 2014; Diner et al. 2016; Xu et al. 2016; Wang et al. 2018), cytomegalovirus (CMV) (Bierle et al. 2016), Epstein–Barr virus (EBV) (Kanda et al. 2016), and Kaposi's sarcoma herpesvirus (KSHV) (Avey et al. 2015; van Diemen and Lebbink 2017).

Annually, a huge sum of money is dissipated because of the loss of agriculturally important crops owing to viral infections. This matter is, therefore, a serious hurdle in assuring food security for the growing world population (Andolfo et al. 2016). A possible solution to this is to engineer the genome of host plants so as to improve their resistance against plant viruses (Khatodia et al. 2017). The CRISPR-Cas9 technology has the potential to serve as a novel antiviral agent for the protection of plants (Zhang et al. 2015). The CRISPR-Cas9-mediated virus resistance is broadly divided into two approaches: one is where the viral factors concerning the viral genome are targeted, while the other is where the host factors involved in supporting the viral cycle are meant to be targeted. However, using the CRISPR-Cas9 to target viral genes has been so far restricted just to the model species *Tobacco* and *Arabidopsis* (Khatodia et al. 2017).

Introducing the mutations at the attacking site of the virus through CRISPR-Cas9 protects the herbaceous plant *Nicotiana benthamiana* against the beet severe curly top virus (BSCTV) (Ji et al. 2015). Similarly, the resistance to bean yellow dwarf virus (BeYDV) has been also achieved by specifically knocking out the viral replication initiator protein (*Rep*) gene in transgenic *N. benthamiana* plants (Baltes et al. 2015). A broad spectrum resistance to a series of geminivirus including the tomato yellow leaf curl virus (TYLCV), beet curly top virus (BCTV), and Merremia mosaic virus (MeMV) is possible on Cas9-gRNA-mediated editing of the viral coat protein genes, Rep protein, and its conserved intergenic region (IR) (Ali et al. 2015). Thus, it can be said that CRISPR-Cas9 system has presented a number of ways to eradicate animal and plant viruses. However, with the limitless interactions of macromolecule found in the nature, more studies about them would definitely help to favor the efficient removal of plant pathogenic virus through Cas9-dependent arsenals.

12.5.3 Genome Editing of Parasites Using CRISPR-Cas9 System

The CRISPR-Cas9 tool has been implemented for the genome editing of a number of parasites including *Toxoplasma gondii* and *Plasmodium falciparum* (Ghorbal et al. 2014; Kuang et al. 2017; Payungwoung et al. 2018). On the other hand, CRISPR-Cas9 has made its way into the genome editing of *Trypanosoma cruzi* and *Leishmania*. Expressing the Cas9 endonucleases under the control of dihydrofolate reductase–thymidylate synthase (DHFR-TS) promoter and placing sgRNA under the direct control of U6snRNA promoter and terminator give rise to null mutants in *Leishmania* parasites (Sollelis et al. 2015). Another popular example is the Chagas disease-causing *T. cruzi* (Bern et al. 2011). These parasites spread through the biting of insects called Triatominae, commonly known as "kissing bugs." Knocking out genes (*Pfr1*, Pfr2, and *Gp72*) that are the key components of this particular parasite's flagellum revealed their association with flagellar attachment and cell motility (Lander et al. 2015). Repressing the expression of β-galactofuranosyl glycosyltransferase family of enzymes by multiplexing CRISPR-Cas9 in *T. cruzi* is another approach to reduce the outcome of the enzymatic product. Such kind of CRISPR-Cas9-based approaches may help to determine the drug and vaccine targets designed against kinetoplastid parasites (Chiurillo et al. 2017).

Another classic example of CRISPR-Cas9-mediated pest control is of *Plutella xylostella*. Popularly known as Diamondback moth, *P. xylostella* is responsible for damaging cruciferous crops (cauliflower, broccoli, cabbage, Brussels sprouts, etc.). Targeting the abdominal-A moth gene (*Pxabd-A*) involved in characterization and functioning of the abdominal segment results in inheritable defects and malformation of appendages in both sexes (Huang et al. 2016). Currently, CRISPR-Cas9 is less explored in plant pathogens. However, it can be further expanded in a wide range of plant pathogens such as fungus, bacteria, and viruses for controlling and managing diseases that allow us to improve the crop productivity.

12.5.4 CRISPR-Cas9 System for Removal of Bacteria

The Gram-negative bacterium *Escherichia coli* is among the most extensively studied organism from the genome editing perspective. *E. coli* is associated with 70–95% of urinary tract infections (UTIs), delineating its pathogenesis by forming a biofilm on the inner surfaces of the indwelling urinary catheter (Kucheria et al. 2005). Another member of the *Enterobacteriaceae* family, *Klebsiella pneumoniae*, is known to behave in a similar manner as *E. coli*. Both the uropathogenic strains of *E. coli* and *K. pneumoniae* trigger the catheter-associated urinary tract infections (CAUTIs), which is a very common nosocomial infection. The ability of these microbes to form biofilm over biotic and abiotic surfaces is principally regulated through a phenomenon called quorum sensing (QS). QS is the mechanism in which the bacterium establishes the cell-to-cell communication, senses the bacterial population, and regulates its gene expression accordingly (Rutherford and Bassler 2012; Gohil et al. 2018).

Once the QS mechanism is activated, the bacteria release the signaling molecules called autoinducers (AIs) into the intra- and extracellular environment. Once a threshold of AIs in the extracellular environment is reached, the microbes upregulate the biofilm formation or shape their protein expression accordingly (Sturbelle et al. 2015). To control this interaction, the *Lux* family of genes has been targeted through the CRISPR-Cas9 system. Of the many QS pathways involved, such as LuxR-SdiA, LuxS/AI-2, AI-3, and indole system, the LuxS/AI-2 system is reported to be directly linked to the central metabolism of *E. coli*, while the AI-2 is known to be involved in initiating the biofilm formation (De Keersmaecker et al. 2006). A precise deletion of the involved AI-2-dependent *LuxS* gene through CRISPR-Cas9 tool results in the downregulation of biofilm production (Kang et al. 2017).

The broad spectrum antibiotics tend to relentlessly kill the gut commensals. A possible solution to this problem is to design RNA-guided nucleases that distinctly target DNA sequences matching the organism of interest. The sgRNA-driven CRISPR-Cas9 plasmid, introduced into the bacterial population via bacterial or bacteriophage-based delivery, is designed in such a manner that selectively knocks down any of the undesirable gene, which may include the genes that confer virulence or those involved in antibiotic resistance. The tool works well for targeting the carbapenem-resistant *Enterobacteriaceae* as well as enterohemorrhagic *E. coli* (Citorik et al. 2014). A similar example of programmable removal of microbe, particularly *Staphylococcus aureus*, has been achieved by targeting sequence-specific guide-RNA-mediated antimicrobial action of CRISPR-Cas9 that snips off the targeted virulence genes in the virulent strains, leaving untouched the avirulent strains (Bikard et al. 2014). More often than not, the antibiotic-resistant genes reside within the inherent plasmids and are transferred between the strains through the exchange of such promiscuous plasmids. The abovementioned CRISPR-Cas9 system specifically targets and destroys the staphylococcal plasmids bearing antibiotic-resistant genes and prevents its spread among the avirulent staphylococcal strains (Bikard et al. 2014).

So far, the employment of the gRNA driven Cas9-mediated removal of bacterial pathogens that infect plants has been limited. However, by referring to the above examples, it can be stated that the Cas9 shows the potential of eliminating the undesired pathogens or their toxic and virulence property from a given environment. It would take time to reach at a reliable stage as few barriers need to be overcome. Delivery of the antimicrobial Cas9 vectors and the bacterial resistance (Pursey et al. 2018) against such vectors are the example of barriers that are to be conquered and require thoughtful consideration.

12.6 Conclusion and Future Remarks

Since its development from the early 2013, the CRISPR-Cas9 technique has been applied to a vast variety of biological studies. Compared to conventional transgenic techniques, CRISPR-Cas9 is undoubtedly an accurate and constructive way of genome editing. Therefore, employing the CRISPR-Cas9 system to manipulate the

genome of the model strains may help to overcome the pathogenicity and multiplication of the targeted organism or to derive important bioactive compounds from them. This may in turn help to speed up either the development of programmed novel strains with improved efficiency or to knockout undesirable genes. Even though gRNA-mediated genome correction is in its infancy for some microorganisms, this technology definitely promises a better future with functional benefits. The CRISPR-Cas9 system has a great future ahead in plant biotechnology for controlling microbial pathogens and allowing one to solubilize the complex nutrients into a simpler form which can be easily made available to plants in order to increase productivity and yields.

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