

Chapter 17

CRISPR-Cas systems as antimicrobial agents for Agri-food pathogens.

Gacem Mohamed Amine^{*1}, Hiba Gacem², Djoudi Boukerouis^{1,3}, Joachim Wink⁴

¹. Department of Biology, Faculty of science, University of Amar Tlidji, Laghouat 03000, Algeria.

². Epidemiology Service and Preventive Medicine, Department of medicine, Faculty of Medicine, Hassani Abdelkader University Hospital Center, University of Djillali Liabes, Sidi-Bel-Abbes 22000, Algeria.

³. Applied Biochemistry Laboratory, Department of Biology, Faculty of science, University of Bejaia, 06000, Bejaia, Algeria.

⁴. Working Group Microbial Strain Collection, Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany.

Corresponding author e-mail:

biologieamine@yahoo.fr*, boukerouisdjoudi@yahoo.fr, hiba_1_7_g1991@yahoo.fr, Joachim.Wink@helmholtz-hzi.de.

Abstract

The CRISPR-Cas systems identified in microorganisms are very diverse in their functional organization and mechanism. They participate in the illustration of the development of the strain that carries them over time. The reason is that: each time an exogenous genetic fragment is encountered, a spacer is acquired and then inserted into the CRISPR array. These spacers can provide very important historical information on external aggressors, their ecology and geography. This complex arrangement has a dynamic protection and defense capacity against exogenous genetic elements, which gives the bacterial cell better protection. On the genomic level, the understanding of its functional mechanism is a key factor in its exploitation as a molecular biology and genome editing tool. This chapter aims at clarifying the applications of the CRISPR / Cas system in the preservation and security of crops and food against viral, fungal and bacterial alterations.

Key words: CRISPR / Cas, exogenous, genetic elements, genome editing.

17.1. Introduction

Good nutrition and a well-diversified diet are among the conditions for a healthy and active life (Ochieng et al., 2017). To do this, the world's population has undergone major changes in diets. It has moved towards a diet based on a more diversified diet. This transition is the result of socio-demographic change, trade liberalization policy, urbanization and the marketing of the food industry (Kearney, 2010). The international meeting organized by the Food and Agriculture Organization (FAO) of the United Nations and the World Health

Organization (WHO) also declared that there is a change in the world diet, even in underdeveloped countries, where per capita availability increased by 10%. This transition is the result of significant consumption of livestock products, processed food, fast food, economic growth, and liberalization of investments (Traill et al., 2014).

The use of beneficial bacteria in food manipulations which aim to create a variety of products has been practiced since a long time. This manipulation is passed on from generation to generation. Currently, many microorganisms are used in controlled transformation and fermentation processes adapted to production systems on an industrial scale (Melini et al., 2019). These fermented food products are widely consumed because of their improved sensory and nutritional properties. They contain a high potential of antioxidant substance, antihypertensive peptides, vitamins and other constituents. Furthermore, the availability of living microbes in fermented foods offers more benefits for consumer health (Rezac et al., 2018; Melini et al., 2019). However, the alteration of foods by the pathogenic flora leads in most cases to food poisoning, while in certain cases they can be disastrous as reported in several surveys. In Iran in 2017, researchers were able to isolate toxin-A from a traditional cheese after a foodborne illness from a family with symptoms of botulism (Faghih Solaymani et al., 2019). The same disease is reported in the USA in California after ingestion of cheese sauce. The *Clostridium botulinum* producer of BoNT / A and / or BoNT / A have been detected in patients with symptoms of food botulism (Rosen et al., 2019). With around 600 million people suffering from a foodborne illness, it has become a very alarming global public health problem. This has obliged several countries to modify their constitutional regulatory laws in particular: food control management; inspection services; laboratory services; food monitoring and epidemiological data (Faour-Klingbeil and C D Todd, 2019). With the progression of microbiology and molecular microbiology tools, researchers are well aware of the importance and danger of bacteria in food intended for human consumption. These tools have also disclosed the critical role that bacteria induce in fermented products in modifying tastes, texture, composition, etc. (Waters et al., 2015). The tools of molecular biology have made it possible to explain and understand the mechanism of probiotics on the modification of the intestinal microbiota, the strengthening of the intestinal epithelial barrier and the stimulation of the immune system by offering a natural remedy without additives (Bermudez-Brito et al., 2012). Thanks to “next-generation sequencing”, a large variety of isolated and studied bacterial strains are used in large-scale industrial fermentation processes (Bergsveinson et al., 2017). Molecular techniques have also revealed the mechanism of action of pathogenic bacteria and their toxins

and virulence factors responsible for food and crops spoilage and foodborne illness, because the characterization and understanding of pathogenicity strategies and mechanisms are essential keys in the fight against bacterial diseases (Wilson et al., 2002).

These advances in genomics and molecular biology have forced the food industry to deliver healthy and safe products. This crucial point can only be reached if the microbiota of the food product is controlled throughout the manufacturing process and during storage. Food safety is only the first step in manufacturing, as the industry must also preserve the beneficial microorganisms incorporate in fermented foods from the reaction of harmful pathogens and from their deterioration. One of the advanced scientific wonders of microbiology and molecular biology has been the discovery clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated sequences (Cas) in archaea and bacteria. The first suggestion of the existence of CRISPR occurred in 1987 in *Escherichia coli* during an analysis of the genes responsible for the metabolism of phosphates. The researchers detected unusual repetitive DNA arrangements. Subsequently similar sequences have also been discovered in other bacteria and halophilic archaea (Ishino et al., 2018). After this discovery, studies on CRISPR underwent exponential evolution, due to the capacity of this molecular tool to perform specific DNA cleavage and its potential in editing genomes. Currently, this tool is applied in various medical, biotechnological and agricultural fields (Adli, 2018). In 2014, several research teams demonstrated the performance of CRISPR / Cas in the elimination of resistance genes in bacterial communities (Pursey et al., 2018). Furthermore, the application of CRISPR in the food industries is also enormous for improving the results of starter cultures and probiotics, eradicating harmful microorganisms and spoilage pathogens (Stout et al., 2017). CRISPR / Cas-9 was also applied in plant genome editing, specially on genetic model species (Shan et al., 2020). This chapter treats precise details regarding CRISPR / Cas and its mechanism as a new technique for managing pathogenic microorganisms responsible for spoilage in agriculture and food.

17.2. Role of CRISPR / Cas system in bacterial immunity

17.2.1. Structure of CRISPR in bacteria

The term CRISPR was used for the first time in 2002 (Jansen et al., 2002). As previously described, CRISPR was revealed in *E. coli* 32 years ago. In 1993, repetitive sequences are also detected in two genomes of *Haloflex mediterranei* (Mojica et al., 1993). After more than 7 years, the Mojica team has demonstrated that CRISPR is the most extensively dispersed family

of repetitions in prokaryotes (Mojica et al., 2005). The CRISPR array and the Cas genes protect bacteria and archaea which contains them in its genome against invasive genetic portion by transduction, transformation or conjugation, via a rupture of foreign DNA or RNA fragments. CRISPR / Cas complex therefore constitutes an immune system. It can neutralize and restrict the propagation of antibiotic resistance in pathogenic microorganisms by targeting DNA and preventing conjugation and plasmid transformation as well (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). CRISPR sequences are located in the genome near the genes Cas (Hille and Charpentier, 2016). They are composed of 25 to 50 bp separated by unique sequence spacers of similar length (Bolotin et al., 2005). The CRISPR spacers integrated into the CRISPR locus are derived from a preexisting sequence either of chromosomal origin or of transmissible genetic elements (bacteriophages or conjugated plasmids) (Mojica et al., 2005). The CRISPR is composed of a leader sequence (LDS) which plays the role of promoter in the signals of transcription and initiation of adaptation. The LDS is followed by a series of repetitions installed between the spacers (Alkhnabashi et al., 2016). Bolotin and his group have suggested that the spacers are traces of former invasions by extrachromosomal elements. They are involved in cellular immunity (Bolotin et al., 2005).

17.2.2. Arrangement of CRISPR / Cas type system

The multiplication of research carried out on CRISPR / Cas in recent years has led to the detection of new, very diverse CRISPR / Cas systems. These findings considerably clarify the functional diversity of these operations characterized by an extraordinarily varied architecture (Makarova et al., 2015). Makarova and her team have suggested a polythetic classification which includes the phylogeny of the most common Cas genes, the sequence and arrangement of CRISPR repeats and the architecture of CRISPR / Cas loci (Makarova et al., 2011). The last updated classification of CRISPR / Cas systems published by the Makarova group includes 2 classes, 6 types and 33 subtypes (Table 17.1), against 2 classes, 5 types and 16 subtypes published by the same group in 2015 (Makarova et al., 2019). This classification is based mainly on the diversity of the Cas proteins forming the operon and the sequence dissimilarity between the effector modules (Makarova et al., 2011; Makarova et al., 2018).

Insert Table 17.1. here

Table 17.1. Classification of CRISPR (clustered regularly interspersed short palindromic repeat) -Cas (CRISPR-associated) systems.

The table is organized into two classes (purple), six types (blue) distinguished by the presence of a signature protein and a subtype (different colors) distinguished by the organization of CRISPR loci, the presence or inactivation of additional Cas genes and variation of Cas genes via types.

17.2.3. Functioning mechanism of CRISPR and Cas proteins and their proposed role

Despite this large variance between the types of CRISPR / Cas systems, their functioning principle against exogenous invasive nucleic elements is mediated by a fundamental process.

[Figure 17.1](#) explains the natural mechanisms of the microbial system in adaptive immunity.

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Fig 17.1. Natural mechanisms of CRISPR / Cas systems in microbial adaptive immunity.

After the infiltration of the bacterial cell by extraneous genetic portions, the CRISPR / Cas system decomposes the portions introduced into the bacteria and installs the spacer sequences in the CRISPR locus in the genome through three crucial stages (adaptation, expression, interference); 1) Recognition and acquisition of exogenous spacers by certain enzymes associated with CRISPR (Cas-1 and Cas-2) and installation of spacer sequences in the CRISPR locus in the genome. The spacers are separated between repetitions on the genome and allow the CRISPR to mediate recognition of self and non-self; 2) Transcription of a non-coding RNA (pre-crRNA) by RNA polymerase (RNAP). This pre-crRNA is matured enzymatically and then cleaved into small crRNA by CRISPR-associated specific ribonucleases and gives rise to several small crRNAs. The crRNA is unique for each category of CRISPR system. These crRNAs are also named guide RNAs; 3). Interference is the final step that agrees crRNAs to recognize foreign DNA or RNA with high complementarity, which subsequently leads to a decomposition of the complex formed ([Hsu et al., 2014](#); [Shabbir et al., 2019](#)).

[Stretch and Chertow \(2019\)](#) have well explain the steps of immunity via CRISPR / Cas in bacteria. During the adaptation phase, the foreign genetic material is acquired by the bacteria. The proto-spacers are selected then the spacers are integrated into the CRISPR array. For example, in the type I-E, the Cas-1 and Cas-2 proteins recognize the proto-spacers-adjacent motives (PAM), then breaks them. Another protein independent of CRISPR, known as integrated host factor (IHF), folds the DNA and the complex (Cas-1 and Cas-2) integrates it according to the AT-rich leader sequence in the CRISPR array. For subtype II-A, Cas-9, Csn-2, and tracrRNA are needed for the acquisition of spacers. Cas-1/Cas-2 directs the spacer and recognizes the leader-anchoring site (LAS). The mature crRNA employed by the microorganism to guide the Cas to their targets is obtained after the transcription of pre-crRNA which comprises a multiple repetition and spacing fragment afterwards undergoing cleavages. The maturation system varies from one type to another and even between the subtypes. For

example, in type I (class-I), Cas-6 breaks the pre-crRNA and generates a complex associated to the Cas protein (crRNA-Cas-6). In class-II, the maturation of pre-crRNA is achieved by non-Cas proteins and Cas proteins (Cas-9, transactivating-crRNA (tracrRNA) and RNase III). These proteins are also used in interference. In others types of class-II, the maturation of pre-crRNA is realized by Cas-12 and Cas-13 proteins, respectively (Strich and Chertow, 2019).

The cleavage mechanism of foreign genetic material differs between the subtypes of class-I. For example, type I is formed from a compound complex (Cascade components) where Cas-6 and Cas-5 bind to crRNA, Cas-7 arranges the backbone, while Cas-8 determines PAMs on the target of foreign DNA, Cas-3 cleaves the DNA strand. In the class-II, the interference complex is exchanged by the Cas-9 nuclease guided to the target DNA by crRNA and tracrRNA. Once the complex recognizes the target DNA, Cas-9 cleaves the double strand of target. The subtypes of this class recruit other proteins, such as Cas-12, Cas-13 nuclease and effector proteins (Strich and Chertow, 2019).

17.3. The CRISPR / Cas-9 system and its utilization in genome editing

The efficiency of the CRISPR / Cas system in plants is affected by the structure of single guide RNA (sgRNA) and its expression level, the codons of Cas-9 and its expression level, and the organization of target DNA. This requires major precautions during production. The introduction of sgRNA and Cas-9 into plants may be accomplished by *Agrobacterium*-mediated transformation (Bao et al., 2019), or by agroinoculation (Zhang et al., 2018), particle bombardment (Shi et al., 2017) and PEG-mediated transfection (Anderson et al., 2017). The promoters used for the expressions of the Cas and sgRNA genes are also a crucial part for the progress and success of the tool in plants. The most used promoters for Cas-9 are 35S and ZmUbi, and for sgRNA are AtU6, TaU6 and CaMV 35S (Zhang et al., 2018; Macovei et al., 2018; Jia et al., 2017).

Under certain circumstances, Cas-9 cleaves non-target DNA. In order to limit these off-target mutations, the researchers developed high-fidelity Cas such as SpCas-9-HF1 and eSpCas-9 (Kleinstiver et al., 2016; Slaymaker et al., 2016). In addition, the researchers invented tools such as CRISPR-P 2.0 for editing the genes in plants in order to better develop the CRISPR system (Liu et al., 2017). The two most regularly applied genome editing methods are Non-Homologous End Join (NHEJ) and Homologous Recombination (HR). CRISPR / Cas-9 is used in agriculture to edit the genome of plants in order to improve nutritional trait as in the case of bananas (Kaur et al., 2020), or in soybean to modify the fatty acid and protein levels (Wu et al.,

2020). CRISPR / Cas-9 is applied in potato (*Solanum tuberosum L.*) to target StPPO2 gene in order to decrease polyphenol oxidases, an enzyme responsible for enzymatic browning in fruits and vegetables (González et al., 2020). in Ryegrass (*Lolium spp.*), the CRISPR tool is applied to improve the rust resistance, and spring growth, because this culture is used in milk and meat production (Zhang et al., 2020). The application of CRISPR tool in rice for three genes editing namely; OsPIN5b, GS3 and OsMYB30 demonstrated that new rice varieties have high yield and excellent cold tolerance (Zeng et al., 2020). In this chapter, we are interested in the applications of CRISPR / Cas in plants in order to increase tolerance and resistance to biotic stresses.

17.4. CRISPR-Cas systems application in food, agri-food and plant

Food is the source of energy and the basis of mineral elements and nutrients for all living things. Certain vegetable-based foods, animal products and beverages produced by fermentation represent 10 to 40% of the overall diet with a cultural heritage of high gastronomic value (Talon and Zagorec, 2017; Sivamaruthi et al., 2018). The fermentation process has long been applied to protect, preserve and develop the quality of the fermented food. The microorganisms used in the production of this food also contribute to the functional properties of the food. These types of food have an impact on human cognitive function (Sivamaruthi et al., 2018). The functional characteristics which define these fermented foods are related to probiotic bacteria where the microbial communities produce bioactive molecules such as bioactive peptides for example, or by the addition of non-microbial functional compounds (Leroy and Vuyst, 2014). The health role of functional fermented food is more important than we imagine. This food can modify the intestinal physiology which in turn influences biological mechanisms, for example; prevention of depression and anxiety (Aslam et al., 2018).

The starter cultures and the probiotics bacteria employed in the fermentation manipulation have other advantages: 1) - They oppose the pathogenicity of pathogenic bacteria such as for example; the prevention of the adhesion of *Listeria monocytogenes* by *Lactobacillus rhamnosus* CTC1679, *Lactobacillus sakei* CTC494 and *Enterococcus faecium* CTC8005 on intestinal epithelial cell line HT29 (Garriga et al., 2015), 2) - Reduction of food losses and enhancement of product safety by elimination of biogenic amines and mycotoxins (Laranjo et al., 2019), 3) - Improvement of the quality of the fermented product with characteristic sensory properties (Rubio et al., 2014), 4) - Development of the organoleptic quality of the fermented food by glycolysis, lipolysis, proteolysis, and the production of many compounds such as

organic acids, polyols, exopolysaccharides, antimicrobial and bacteriocins compounds (Bintsis et al., 2018), 5) - Food fermented by its antioxidant activity and its composition can prevent cardiovascular disease, cancer, gastrointestinal disorders, allergies, and diabetes (Tamang et al., 2016).

Although food fermentation process employs only beneficial microorganisms, this procedure suffers from numerous difficulties, some of which are worrying, such as the occurrence of mycotoxin and toxins of bacterial origin (Sivamaruthi et al., 2019), and the failure in fermentation due to infection of beneficial bacteria by bacteriophage (Marcó et al., 2012; Pujato et al., 2019). These problems encountered during fermentation as well as the improvement of starter cultures are the main worries of any food industry. CRISPR / Cas is a new tool for good management of beneficial bacteria and limiting the occurrence of pathogens (Stout et al., 2017). On one hand, a study carried out on 1262 genomes of lactobacilli revealed the prevalence of CRISPR / Cas in the genomes studied, on the other hand, their contents vary between the strains (Crawley et al., 2018). This CRISPR / Cas based technology suggests a wide range of advantages for designing *Lactobacillus* and *Bifidobacterium* to improve gene expression and provide new functionality (Hidalgo-Cantabrana et al., 2017a). It makes it possible to edit the genome (Leenay et al., 2019), to improve the resistance of bacteria to phages (Watson et al., 2018), and to use it as an antimicrobial agent (Pursey et al., 2018).

17.4.1. The benefit of CRISPR / Cas systems in starter culture preparation

The fermentation industry needs very effective in-depth molecular methods and techniques to identify strains of interest, and / or harmful bacteria, or to enhance the functionality of typical strains already used in the manufacturing process. Several techniques are used such as for example; pulsed field gel electrophoresis, repetitive extragenic palindromic, and multiplex polymerase chain reaction (Adzitey et al., 2013). In the food manufacturing, the selection of strains forming the starter cultures is a decisive step for successful fermentation. Sequencing of 16S rDNA, DNA-DNA hybridization, G + C percentage, characterization of secondary metabolites and genes exploration by bioinformatics are all cost-effective practices for characterizing strains. The discovery of CRISPR / Cas and the characterization of its dynamic aspect create a new way of typing the strains of interest by sequencing the CRISPR / Cas array. It is a new favorable tool which makes it available to identify the strains and to study the links of kinship and divergences between the strains. Briner and Barrangou have demonstrated that type II-A CRISPR / Cas systems is effective for the genotyping of

Lactobacillus buchneri. The results of this research carried out on 26 isolates of *L. buchneri* demonstrated the presence of 10 unique genotype loci containing CRISPR arrays that covering a CRISPR locus of 36 nucleotides of type II-A. Characterization demonstrated the presence of conserved spacers, and polymorphisms reflecting a recent divergence (Briner and Barrangou, 2014). The genotyping of lactic acid bacteria and probiotics of industrial interest is very effective in these genera because lactic acid bacteria harbor a varied set of CRISPR / Cas systems (Sanozky-Dawes et al., 2015). *S. thermophilus* CRISPR / Cas system (StCas) presents a great diversity and excellent capacities of integration of exogenous DNA. In addition, the existence sgRNA / Cas-9 nucleases, makes this model of CRISPR an effective tool of genomic modification (Hao et al., 2018). The genus *Bifidobacterium* which is known by these beneficial effects contains a large diversity of species, some of which, like *B. longum*, contain a variety of CRISPR / Cas systems in their genomes, in particular Type I (I-C, I-E) and Type II (II-C) (Hidalgo-Cantabrana et al., 2017). CRISPR / Cas genotyping is an effective molecular tool for characterizing microorganisms of industrial interest by disclosing valuable information on their phylogeny, evolution and ecology (Stout et al., 2017).

17.4.2. Development of CRISPR / Cas-9 against virus resistance in agriculturally crops

Climate change, the increase in the use of pesticides and the development of microbial resistance to biocides are influencing the emergence of phytopathogenic diseases. These pathologies have a significant impact on significant crop losses (Lechenet et al., 2017; Sundin and Wang, 2018; Velásquez et al., 2018). By their emergence and their important toxicity, the practice of pesticides states an immense problem for environmental ecosystems (Lushchak et al., 2018). Furthermore, their phytotoxicity is another problem that makes it very complicated to control phytopathogenic microorganisms (Lalancette and McFarland, 2007; Kumari et al., 2019). Currently, the management of these pathogens is mainly based on prevention through the use of bactericides (copper and antibiotics), biological control through the use of beneficial bacteria and genetic modifications (Sundin et al., 2016; Daranas et al., 2019; Dong and Ronald, 2019).

Field crops are subject to numerous viral infections which make them ranked in the same rank of health and economic danger caused by fungal and / or bacterial alterations. In order to protect food safety against viral alterations, researchers have thought of enhancing the resistance of field crops to diseases. But this solution remains far from the desired results following the rapid evolution of viruses. CRISPR / Cas-9 has become a very effective molecular

biology tool making it possible to enhance plant resistance to viruses either by eradicating viruses by targeting viral factors in the virus, or by modifying factors favoring development of virus in the host plant by plant genome editing (Khatodia et al., 2017). This molecular tool can be also applied in the study of different physiological processes in plants (Nguyen et al., 2020). CRISPR / Cas-9 tool against plant viruses is practiced for the first time in 2015 against Geminivirus in *Nicotiana benthamiana* (Ji et al., 2015). The same group of researchers has developed a transgenic *N. benthamiana* and *Arabidopsis thaliana* capable of expressing Cas-9 and sgRNA. Transgenic plants have divulged a blockage of viral accumulation by mutations in Beet severe curly top virus (BSCTV) with eradication of the symptoms of viral infections. In the same year, another group demonstrated the efficacy of CRISPR / Cas-9 as an antiviral agent against Tomato yellow leaf curl virus (TYLCV) into *N. benthamiana* (Ali et al., 2015). This genome editing system modifying the resistance of plants against viruses may be widely applicable in the next future since deep sequencing detects no effects outside the viral target (Ji et al., 2018).

The CRISPR / Cas system is also capable of targeting RNA viruses. C2c2 (Class II / type VI, (Cas-13a)) got from *Leptotrichia shahii* is capable by its RNA-guided ribonuclease function of interfering with RNA phage (Abudayyeh et al., 2016; Abudayyeh et al., 2017). The design of CRISPR / Cas-9 composed of FnCas-9 obtained from *Francisella novicida*, and sgRNA intended to fight the Cucumber mosaic virus (CMV-RNA virus) in transgenic *Arabidopsis* demonstrated the absence of symptoms of viral infections. In addition, the Elisa test and RT-qPCR have shown that viral infection is inhibited (Zhang et al., 2018). Some studies have proposed to widen the applications of CRISPR / Cas-9 on a wide variety of culture viruses, such as for example; Iqbal and his group who proposed a multiplexed CRISPR / Cas-9 system in order to combat the Begomoviruses (DNA virus) responsible for the Cotton leaf curl disease (CLCuD) associated with satellite molecules called alpha- and beta-satellite (Iqbal et al., 2016). The design and translocation of another CRISPR / Cas-9 in *N. benthamiana* and targeting the TYLCV has demonstrated that sgRNA exerts excellent interference and cleavage at the level of the stem sequence- loop within the origin of TYLCV replication in the intergenic region (IR) (Ali et al., 2015). Targeting non-coding intergenic regions causes interference, with ineffective recapture of mutated viral variants. This stops the regeneration and replication of variants (Ali et al., 2016). CRISPR / Cas-13a mediated by *Agrobacterium* carrying TRV vector in *N. benthamiana* is effective against the helper component proteinase (HC-Pro), GFP sequences and coat protein (CP) sequence of Turnip mosaic virus (TuMV) (Aman et al., 2018). Figure

17.2 demonstrates the development of plants resistant to viruses by targeting viral factors in the virus by CRISPR / Cas genome editing.

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Fig 17.2. Development of plants resistant to viruses by targeting viral factors in the virus by CRISPR / Cas genome editing.

As described above, in order to counter viral infection, it is also possible to concentrate on the plant genes encouraging the development of the viral cycle as it is to eradicate the virus itself (Khatodia et al., 2017). This molecular tool is designed to be an adequate and more sustainable approach against phytopathogens. But before going to this stage, it is necessary to know the recessive resistance genes (RRG) against viruses. There are currently 14 natural RRGs, 12 of which encode the eukaryotic translation initiation factor 4E (eIF4E) or its isoform eIF(iso)4E (Wang and Krishnaswamy, 2012). In 2014, Zhang and his group unveiled 11 genes in rice with the ability to be edited by CRISPR / Cas-9 with high efficiency and the absence of any new mutation in the first generation (Zhang et al., 2014). The disruption eIF4E factor in *Cucumis sativus* by sgRNA / Cas-9 which targets the N 'and C' terminal ends of the eIF4E genes demonstrated a small deletion and unique mononucleotide polymorphisms in the target genes of the first generation. Remarkable antiviral resistance has been demonstrated against vein yellowing virus (Ipomovirus), Zucchini yellow mosaic virus and Papaya ring spot mosaic virus-W in non-transgenic heterozygous plants (Chandrasekaran et al., 2016). The introduction of specific deleterious point mutations at eIF(iso)4E locus in *A. thaliana* by CRISPR / Cas-9 tool leads to total resistance to TuMV (Pyott et al., 2016). In rice (*Oryza sativa*), the generation of mutations in eIF4G by CRISPR / Cas-9 machinery has proved remarkable resistance of the plant to Rice tungro spherical virus (RTSV) (Macovei et al., 2018). This genomic tool will completely remove the genes necessary for the development of viruses in the host and establish a spectrum of permanent resistance. Figure 17.3 reveals the evolution of plants resistant to viruses altering factors favoring development of virus in the host plant by plant genome editing.

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Fig 17.3. development of plants resistant to viruses altering factors favoring development of virus in the host plant by plant genome editing.

17.4.3. Development of CRISPR / Cas-9 against fungal resistance in agriculturally crops

Fungal pathogens are known for their irreversible disaster in the fields and in storage rooms. They are responsible for several fungal diseases in agricultural crops. This results in significant economic losses. These harmful effects are not limited to that on crop losses, mycotoxinogenic molds are capable of secreting mycotoxins whose toxic effects are extremely dangerous for human and animal health (Gacem and Ould El Hadj-Khelil, 2016; Borrelli et al., 2018). Several strategies based on molecular biology and genetic modification have been applied to improve the resistance of plants to phytopathogenic agents. Several types of genes have been modified and introduced into the plant in order to fight harmful fungal agents. Currently, these genetic manipulations of plants are based on CRISPR / Cas as a new molecular biology tool (Borrelli et al., 2018).

Powdery mildew disease is a one that affects plants. It is caused by ascomycete fungi of the order *Erysiphales*. The discovery of a mutant barley variety capable of resisting "powdery mildew pathogen" (*Blumeria graminis* f.sp. hordei (Bgh)) has shown that the plant has an inherited loss of function mutation in the Mlo gene. The mutation of the Mlo genes in *Arabidopsis* genome also gives it resistance to *Golovinomyces orontii* and *G. cichoracearum* (Acevedo-Garcia et al., 2014). Plants with loss of function alleles at the Mlo locus are resistant to all identified isolates of the widespread powdery mildew fungus (Piffanelli et al., 2004). Another protein called HvBI-1 in barley is a sensitivity factor for powdery mildew. It supports the modulation of the defense of the wall and the penetration of *B. graminis* f. sp. Hordei (Eichmann et al., 2010). In tomatoes (*Solanum lycopersicum* var. *cerasiforme*), the loss of expression of SIMlo-1 also gives it protection against fungal attacks (Bai et al., 2008). The Mlo gene is currently employed to control the majority of varieties grown in Europe in the spring (Piffanelli et al., 2004).

The new molecular biology tool CRISPR / Cas has proved its effectiveness in protecting plants against phytopathogenic fungi. The creation of a mutation in TaMlo-A1 allele in hexaploid bread wheat by CRISPR / Cas-9 led to the generation of a transgenic wheat with high resistance to infection by *B. graminis* f. sp. *tritici* (Wang et al., 2014). The suppression of TaEdr1 in *Triticum aestivum* L also demonstrated good resistance against infection by *B. graminis* f. sp. *Tritici*. Genetic analyzes did not detect any negative effects outside the targeted genomic regions (Zhang et al., 2017). The study of mutations caused in the four genes namely: ZmPDS, ZmIPK1A, ZmIPK and ZmMRP4 from *Zea mays* demonstrated that the CRISPR / Cas tool is also very effective in editing the corn genome (Liang et al., 2014). In rice, the

mutation caused in the Ossec3a and Oserf922 genes by CRISPR / Cas-9 increases resistance to *Magnaporthe oryzae* (Ma et al., 2018; Wang et al., 2016).

In fruits such as tomatoes, the creation of mutations in the SIMlo-1 gene has proved protection against *Oidium neolyopersici* and the suppression of the expression of CaMlo-1 or CaMlo-2 in pepper gives it reduced sensitivity to *Leveillula taurica* (Zheng et al., 2013). Non-transgenic plants such as Tomelo (tomato), obtained by applying CRISPR / Cas-9 which targets SIMlo-1, have shown good resistance to infection by *O. neolyopersici*. Another research has shown that alterations in the tomato SIDMR6-1 gene make it more resistant to infection by *Phytophthora capsici* (Thomazella, 2016).

The application of CRISPR / Cas-9 has also proved its benefits in enhancing the resistance of fruit trees against phytopathogenic fungi. CRISPR / Cas-9 ribonucleoproteins (RNPs) is applied in vine and apple protoplasts in order to create mutations in the Mlo-7 genes, in order to improve the resistance of grapes to powdery mildew, and in the DIPM-1, DIPM-2, and DIPM-4 genes in apples with the aim of improving resistance and fighting fire blight disease (Malnoy et al., 2016). Application of CRISPR / Cas-9 delivered by *Agrobacterium* with four guide RNAs targeting the transcription factor gene VvWRKY52 led to the generation of a transgenic plant, knockout of VvWRKY52 gene improved the resistance to *Botrytis cinerea* (Wang et al., 2018). Other publications have exposed the potential of CRISPR / Cas-9 in protecting plants from fungal attack. Targeting the TcNPR-3 gene in *Theobroma cacao* increases resistance to *Phytophthora tropicalis* (Fister et al., 2018). Disruption of the PpalEPIC8 gene in Papaya and the BnWRKY70 gene in *Brassica napus* augments resistance to infections caused by *Phytophthora palmivora* and *Sclerotinia sclerotiorum*, respectively (Gumtow et al., 2018; Sun et al., 2018). In another study on *Phytophthora sojae*, creating multiple mutations in PsORP1 using CRISPR-Cas-9 demonstrated different resistance levels to oxathiapiprolin fungicide (Miao et al., 2020). Figure 17.4 demonstrates the progress of fungal resistance in transgenic and non-transgenic plant obtained by CRISPR / Cas-9.

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Fig 17.4. Progress of fungal resistance in transgenic and non-transgenic plant obtained by CRISPR / Cas-9.

17.4.4. Development of CRISPR / Cas-9 against bacterial resistance in agriculturally crops

Some studies have brought to light the potential of CRISPR / Cas-9 in the fight against bacterial diseases in agricultural crops. *Y-proteobacterium Xanthomonas oryzae* pv. *oryzae* (Xoo) is a bacterial species responsible for bacterial blight of rice. This bacterium inserts into the plant cell DNA-binding proteins named group of TALEs (the transcription activator-like effectors) that bind to effector-binding elements (EBEs) to induce the expression of the OsSWEET family of putative sugar transporter genes, which work by granting rice sensitivity to pathologies (Zhou et al., 2015; Blanvillain-Baufumé et al., 2017). Xu and his team have identified two PthXo2-like TALEs (Tal5LN18 and Tal7PXO61), virulence factors in strains Xoo, Tal5LN18 and Tal7PXO61 bind to slightly diverse EBE sequences in the promoter OsSWEET13 to activate its expression. The use of CRISPR / Cas-9 to generate InDels in the EBS of OsSWEET13 has led to the generation of Xoo-resistant rice (Xu et al., 2019). Editing the rice genome by a mutation induced via CRISPR / Cas-9 in three genes, namely; host sucrose transporter genes SWEET11, SWEET13 and SWEET14 led to the generation of rice characterized by robust resistance against Xoo (Oliva et al., 2019).

Xanthomonas citri subsp *citri* (Xcc) is a causative mediator of Citrus canker which is a serious pathology that causes enormous losses in Duncan grapefruit. In this fruit, PthA4 is an EBEs which binds to EBEPthA4-CsLOBP to motivate CsLOB1 gene expression. However, in this fruit, there are two alleles, Type I and Type II, of CsLOB1. The inactivation of a single allele by sgRNA / Cas-9 is sufficient to make the fruit tree resistant to infection by Xcc (Jia et al., 2016). Induction of mutations in other genes by sgRNA / Cas-9 has also given good results (Jia et al., 2017), the alteration of EBEPthA4 of the CsLOB1 promoter by CsLOB1sgRNA / pCa-s9 disrupts the expression of CsLOB1 induced by Xcc (Peng et al., 2017). The edition of PthA4 in the Citrus genome (Duncan grapefruit) by the CRISPR-LbCas-12a (Cpf1) resulting from *Lachnospiraceae bacterium* ND2006 led to the formation of transformed plants having a low susceptibility to infection by Xcc (Jia et al., 2019). Figure 17.5 demonstrates the progress of the fight against bacterial resistance in transgenic and non-transgenic plant by CRISPR / Cas-9.

Insert fig 17.5 here

Fig 17.5. Progress of the fight against bacterial resistance in transgenic and non-transgenic plant obtained by CRISPR / Cas-9

P. syringae pv. tomato DC3000 (Pto) produces coronatin (COR) which stimulates the opening of stomata and facilitates bacterial colonization of the leaves. It is also responsible for the tasks on the tomato (Ortigosa et al., 2019). In tomatoes, the modification with small deletions in the SIDMR6-1 gene by the CRISPR / Cas increases the resistance to Pto (Thomazella et al., 2016). The release of SIJAZ2, a major COR co-receptor in tomato cells that controls the dynamics of stomata during bacterial invasion, has demonstrated cell resistance to Pto. The CRISPR / Cas-9 genomic tool made it possible to generate dominant JAZ2 repressors lacking the Jas C-terminal domain (SIJAZ2 Δ jas) (Ortigosa et al., 2019). In apples, *Erwinia amylovora* causes fire blight disease, the specific gene for the disease is DspE. The latter interacts with four serine / theonine kinases similar to similar leucine-rich receptors. These four proteins are coded by DspE-interacting proteins of Malus (DIPM genes) (Borejsza-Wysocka et al., 2006). Mutation induction in DIPM-1, DIPM-2 and DIPM-4 by CRISPR / Cas-9 ribonucleoproteins (RNP) to the protoplast of apple cultivar increases resistance to fire blight disease (Malnoy et al., 2016).

17.4.5. Development of CRISPR / Cas9 against bacterial resistance in food

Bacterial pathogens found in food for human or animal consumption are responsible for a wide spectrum of food-borne diseases and poisoning accompanied by great economic loss as a result of the rejection of food spoiled by bacteria. This worrying situation has led researchers and scientists to develop very effective surveillance systems in order to react as quickly as possible. CRISPR / Cas is a new molecular biology tool capable of being applied in this field. It presents a wide range of powerful applications that can handle the challenges of pathogenicity and bacterial spoilage. For example, brucellosis caused by several species belonging to the genus *Brucella* causes severe zoonotic diseases and consequently, public health problems and substantial financial burdens. A recent study carried out in vitro demonstrated that CRISPR / Cas-9 carried on lentiviral vectors induces a significant decrease in the bacterial load by targeting *Brucella's* RNA polymerase subunit A (RpoLA) or virulence-associated gene virB10 (Karponi et al., 2019). The control of pathogenic bacteria in food by CRISPR / Cas is very complex due to the complexity of the microbial community contained in the food and the method of delivery of the genomic tool. An in vitro study aiming at eliminating *Salmonella enterica* through *Escherichia coli* has shown that the TevSpCas-9 dual nuclease delivered by plasmids based on the IncP RK2 conjugative system results in high conjugation with the cis-acting plasmid and consequently a significant destruction of *S. enterica* (Hamilton et al., 2019). CRISPR / Cas-9 has also been shown to be operative in eradicating mcr-1 gene and restoration of polymyxin sensitivity in *E. coli* (Dong et al., 2019). The insertion of CRISPR / Cas systems

into phages is also capable of destroying virulence genes in bacteria (Park et al., 2017). The CRISPR-Cas plays an important role in the identification and typing of alteration strains during contamination as in the case of *Salmonella* strains (Strich and Chertow, 2019; Li et al., 2018).

17.5. The advantages and limits of CRISPR-Cas systems in agri-food

The most relevant advantages of CRISPR / Cas are its low cost compared to other molecular biology techniques or those using chemical processes or irradiations involved in the modification of the genome. This new genomic tool may be applied in a multiplex, that is to say targeting several genes at the same time. The targeting of genes of interest is based on the use of several sgRNAs. The effectiveness of this tool relies on synthesized sgRNA, on the methods of delivery into the host plant, and on the promoters accompanying the genomic tool (promoters driving the expression of Cas-9 and those driving sgRNAs). Despite the advantages of this technique, CRISPR / Cas can lead to mutations in off-target genes. This problem can be solved by the application of another CRISPR-Cpf1 tool derived from CRISPR. Other limits of use appeared during the evaluation of this system in the fight against phytopathogens where the targeting of the coding nucleotides of different geminiviruses by CRISPR / Cas-9 resulted in the generation of viral variants capable of folding (Ali et al., 2016).

Conclusion and future perspective

Global demographic exposure and the continued growth in demand for agricultural products puts great pressure on agriculture to produce large quantities and with qualities that meet standards. Surveys carried out in recent years have shown that the use of pesticides, some of which are applied massively and in an uncontrolled manner, are responsible for several worrying pathologies without neglecting the appearance of bioresistance in pathogens and uncontrollable environmental pollution. In addition, despite the benefits that genetic modification and improvement have brought, conventional methods have led to significant genetic pollution.

Since its discovery, CRISPR array and the description of its classes, types, subtypes and its Cas proteins, has interested the scientific community for these limitless applications in the various fields of natural science and medicine. It is used in editing the genome and genes. In recent years this molecular tool has oriented agricultural research towards a new pathway producing healthy and GMO-free crops. The CRISPR / Cas system is a promising alternative in the protection of cultures against viral, fungal and bacterial infections. CRISPR / Cas is easy

to apply against phytopathogens, because the molecular mechanisms of infection of these are sufficiently known by scientists. Eradicating the infection requires deletion of a single targeted gene with precision and without damaging other genes. The activation or induction of mutation by the CRISPR / Cas of the target genes must not have any effects on the physiology of the plant and its growth. The application of CRISPR / Cas in the fight against phytopathogens in plants must be accomplished by monitoring the side effects resulting from the multiple resistance obtained. In addition, field tests must be carried out to prove the effectiveness of this new technique in the fight against phytopathogens, and that annual and perennial agricultural crops are kept protected from one year to another.

The success and advancement of CRISPR / Cas technology in improving and protecting agriculture from plant pathogens can only be declared if the protection and resistance to pathogens are sustained over time without negative effects on the plant. The results must be published in order to convince the public who is hostile to this new technique of genetic modification following the failure of conventional techniques and the emergence of resistance to antimicrobial agents.

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