THE JOURNEY OF CRISPR-CAS9 FROM BACTERIAL DEFENSE MECHANISM TO A GENE EDITING TOOL IN BOTH ANIMALS AND PLANTS

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Abstract: *Today we can use multiple of endonucleases for genome editing which has become very important and used in number of applications. We use sequence specific molecular scissors out of which, most important are mega nucleases, zinc finger nucleases, TALENS (Transcription Activator Like-Effector Nucleases) and CRISPR-Cas9 which is currently the most famous due to a number of reasons, they are cheap, easy to build, very specific in nature and their success rate in plants and animals is also high. Who knew that one day these CRISPR discovered as a part of immune system of bacteria will be this much worthwhile in the field of genetic engineering? This review interprets the science behind their mechanism and how several advancements were made with the passage of time to make them more efficient for the assigned job.*

Keywords: CRISPR-Cas9, endonucleases, gene editing, immune system, zinc finger, TALENS

Introduction

It is foreseen that the world's human population will increase to 10 billion by 2050 and with the increasing population the global requirement of food will grow to 25% to 70% of the current production rate (Hunter *et al*., 2017). In order to feed the world it is important to fight against pathogens which are resulting in yield loss of 20-40% around the globe (Savary *et al*., 2012). To overcome the challenges including increase in yield, pathogen resistance, crop improvement it was important to develop some techniques that could replace the conventional selective breeding and induce changes in the genetic makeup of crops. Up till 1970s it was not possible to achieve successful genetically modified organisms. The first transgenic organism that came into existence was by inserting an exogenous DNA into the plasmid of *E. coli* without interrupting any of bacteria's own biological function (Cohen *et al*., 1973). However this was risky process as there were possibilities that the exogenous DNA may interfere with the bacterial DNA and mutate it. There were also the chances of mismatches. Then a technique came, in which the DNA sequence to be delivered were homologous to the sequence that was targeted in the other organism thus giving more specificity and less chances of mismatches. This particular technique was the footstep of gene editing technology (Szostak *et al*., 1983). Its widespread use was limited due to its inefficiency but it was used widely for research purposes. Under the following decades after collecting so much knowledge and after so many studies came the genome editing through programmable endonucleases, which is the most advanced and recent technique. These endonucleases for plants include zinc finger nucleases, transcription effector like nucleases, and CRISPR-Cas9 (Shah *et al*., 2018; Bao *et al*., 2019). CRISPR is a ground breaking tool due to its potential to treat human diseases and edit human genome. It has helped to identify and understand the procedure of diseases (Zhou *et al*., 2014), It has helped generate animal disease models (Xue *et al*., 2014). It has assisted in advancing genetic engineering in crops (Zhang *et al*. 2017). The objective of this mini review is to highlight the features of CRISPR, its advantages and drawbacks over other techniques and to know the reason behind all the fame and publicity and also some of the work done on specific crops.

History and origin of CRISPR

Recently, CRISPR-Cas9 is stealing the show due to their number of attributes. Ishino was the scientist who was analyzing the gene that would conduct isozyme conversion of alkaline phosphatase about 30 years ago and while performing his experiment, he had no idea that he would discover the first ever CRISPRs from Escherichia coli (Ishino *et al*., 1987). By that time it was very mysterious and unexpected, scientist couldn't find out the function of these repeated sequences because the human genome project did not begin its function of sequencing the genome of organisms that have therapeutic importance at that time. Then after being detected in bacteria they were for the first time seen in archaea in

1993, more precisely in Haloferax mediterranei and the man behind the discovery was Mojica who also found out after some years that the function of these sequences found in bacteria and archaea is similar (Mojica *et al*., 1993;2000) Janses named them CRISPR in 2002 (Janses *et al*., 2002) Then four years later Eugene conveyed that the CRISPR-Cas9 system was a prokaryotic RNA interference–based immune system (Makarova *et al*., 2006). In 2007 it was proven practically that they are truly part of the adaptive defensive mechanism of prokaryotes while using the lactic acid bacterium Streptococcus thermophiles (Barrangou *et al*., 2007). In short in 2012 the face of CRISPR was unveiled and by this time scientist knew how they could use this system for cutting the target DNA outside the cell (Gasiunas *et al*., 2012). Then the very next year this system taken from *streptococcus pyogenes* was applied to genome edit human nerve and the kidney cells of mouse (Cong, Mali *et al*., 2013). Beginning an era of targeted genome editing which is easier in handling.

Structure of CRISPR loci

Clustered regularly interspaced short palindromic repeats are found in bacteria and archea as the part of their immune system, they were first analyzed in *Streptococcus pyogenes* and classified as type 2 (Langner *et al*., 2018). Streptococcus pyognenes is known to cause lethal infections in humans from less severe sore throat to a range of infections. It belongs to the gram positive type of bacteria and works by making colonies in the pharynx (part of throat) and skin (Rosinski-Chupin *et al*., 2019). These are a family of DNA sequences consisting of 2 important components, Cas 9 endonuclease and single guide RNA (gRNA) which further consist of pre-crRNA and the trans-activating crRNA also known as tracr-RNA (Weeks *et al*., 2016). Cas 9 endonuclease is a protein and as the name indicates it is an enzyme involved in cleavage, the customizable guide RNA is to escort the enzyme to the site of target sequence (Jiang and Doudna, 2017; Khatodia *et al*., 2016). As the name clearly shows CRISPR consist of short repeats that are 28-37 bp sequences in length (Barrangou *et al*., 2014), these sequences are unique from each other but are of similar length and are separated from each other from spacers. Every repeat is arranged in a palindromic manner. These Spacers play a major role in bacterial immunity as they store the memory of the sequences from all the past attacks. The number of these spacer vary from specie to specie it can be from 1 to 100 (Grissa *et al*.2007). The protospacer adjacent motif (PAM) for streptococcus pyogenes is NGG .The PAM are present downstream of target DNA (Jiang and Doudna, 2017). Further the Cas 9 enzyme consists of two nuclease domain which induces a double stranded break as one domain binds to the target DNA strand which is homologous to the guide RNA and the other domain binds with the non-target strand (Gao *et al*., 2017).

Remodeling CRISPR for genetic engineering

The double stranded break efficiently induced as a result of cleavage activity of Cas 9 enzyme is the main idea behind the diversity in genetic outcomes using these technologies. After the cleavage cellular repair mechanism is activated that is of two types HDR and NHEJ. In case of non-homologous end joining (NHEJ) random DNA pieces are arranged at the end of double stranded break and then are joined by cell's own repair machinery. This method is active in all cycles of cell and is more prone to errors (Moore and Habor 1996). This process does not require homology templates as they directly ligate break ends. They can further cause indels (insertion or deletions) in the region of DSB, this can be used to achieve frameshift mutations (Waters *et al*., 2014). However NHEJ is quite unspecific and random process and is not efficient for single base gene knockout or insertion. Alternatively Homology directed repair occurs when sister chromatids are present. This DNA template contains the Cas 9 and gRNA along with the DNA sequence to be delivered into the cell. This method is more specific and less prone to errors (Pardo *et al*., 2009). However these site specific enzymes are very versatile in nature as in addition to gene editing they are also used as an artificial transcriptional factors. Scientist introduced two mutations in the cleavage domains of cas 9 nuclease which confiscated its cleavage activity but it was still able to bind to the DNA. In this way these systems were used to increase or decrease a gene expression by controlling its transcription (Bikard *et al*., 2013).

Brief discussion of other nuclease

Mega nucleases

Starting with the mega nucleases which are also called homing endonucleases, are site specific like other restriction endonucleases. They have the ability to recognize large target DNA sequence that can be greater than 12 bp (Stoddard 2005). Discovered in 1990 , they have proved themselves to be an efficient tool for genome editing as they can generate homologous recombination (Epinat *et al*., 2003) and desired alterations (Arnould *et al*., 2007). They possess five families out of which most important one is LAGLIDADG (Stoddard *et al*., 2006). As they can induce homologous recombination, they were used in a number of organisms including drosophila, E.coli, mice, plants and trypanosomes (Paques 2007). These mega nucleases are very specific in nature which gives them prescion and show very less level of off targeting effects, cell toxicity but changing the

target of mega nucleases requires a large amount of work to obtain the results (Takeuchi *et al*., 2014; Thyme *et al*., 2014; Smith *et al*., 2006).

Zinc finger nucleases

As we know that zinc finger proteins are widely being used by nature (Bedis *et al*., 2009), so there must be some advantages of its this much use in nature that could be manipulated and reused in the form of artificial enzymes such as zinc finger nucleases. Zinc finger nucleases have been very successful in the field of genome engineering. These extraordinary enzymes consist of customizable zinc protein domain and a cleavage domain of FokI restriction endonuclease (Kim *et al*., 1996). A single zinc finger contains 28-30 amino acids that have a remarkable ability to functionally vary and structural flexibility to bind any triplet depending upon the form DNA sequence (Weirauch *et al*., 2010). These were the first of all the exonucleases that were used in a large amount (Porteus and Carroll 2005; Urnov *et al*., 2010) This genome editing artificial endonuclease has been successfully used in a number of organisms including maize (Shukla *et al*., 2009) Drosophilla (Beumer *et al*., 2008) rat (Geurts *et al*., 2009) most importantly ZFN are in clinical phase trial 2 for the treatments of AIDS proving themselves to be helpful in gene therapy (Cannon *et al*., 2011). But in spite of all these pros, there are always some limitations associated with a technology and as for ZFNs it is their off target effects (Gabriel *et al*., 2011; Pattanayak *et al*., 2011). They are also difficult to construct as compared to more recent and advanced gene editing techniques.

TALENS

Next came the TALENS, these are the proteins found in a bacteria called Xanthomonas that is toxic to plants as it infects them (Moscou *et al*., 2009; Botch *et al*., 2009). TALENs and ZFNs are a bit common in nature as they share some similar features, for instance a cleavage domain of FokI is found in both (Kim 2014). In addition to nuclease domain TALENS consist of a TALE DNA-binding region, which is a repeating unit having about 34 amino acids. The connection between the target DNA and the recognition domain is made possible by the repeat valuable diresidues that are the amino acids present at 12th and 13th position (Boch *et al*., 2009; Moscou and Bogdanove 2009).This exceptional nature and TALEs ability to recognize DNA length of 12-20bps give them specificity in genome editing (Guilinger *et al*., 2014). After some research on these molecular scissors, it came into light that these are more specific in nature and shows less off target effects (Wang *et al*., 2015). Another quality of them making them more acceptable than ZFNs is that there is no need of directed evolution for them thereby saving a

lot of time and experience of combining an enzyme to make it functional (Mussolino *et al*., 2014).

Why CRISPR is preferred over others?

Due to so many reasons CRISPR has replaced ZFN and TALENS. At number of times CRISPR systems have proved themselves more advanced and trustworthy over other techniques (Sander and Joung 2014). For instance ZFNs and TALENs require a customizable protein which will guide them to the target site. The designing and engineering of this protein is complex, time taking and expensive. On the other hand CRISPR depends only on the engineering of short guide RNAs (Sternberg *et al*., 2014). Multiplex gene changing is also very difficult using other two techniques as specific proteins are needed for each gene but by using CRISPR systems multiple gene editing is handy as only many guide RNAs are to be delivered in the cell (Campa *et al*., 2019). Additionally there is this fact that both ZFNs and TALENs work as a dimer to generate a double stranded break at the target site , this can be a problem as loading capacity of some of the vectors is less, so their delivery can be a great hurdle. However the delivery of CRIPSR systems is more obstacles free (Wu *et al*., 2010).

First application of CRISPR in human beings

Previously, genome editing was labor intensive; it could take months, costly and often limited to labs only under the supervision of experts. But now with the emergence of nucleases and advanced gene editing technologies which are friendly, cost effective and takes only weeks, it has become possible to delete the genes, (Lee *et al*., 2010) insert the genes (Moehle *et al*., 2007), replacing the faulty genes (Ran *et al*., 2013) and even rearrangement of chromosomes (Torres *et al*., 2014). Experiments using CRISPR started occurring on other mammals excluding humans. Because experimenting on human beings is always a risky thing to do. In early 2018 a Chinese scientist, Dr. He Jiankui started experimenting to produce babies from embryos that were genetically edited. These were the babies with modifications against HIV. HJ named them Lulu and Nana, In this case a specially constructed CRISPR was injected into the embryo, whose aim was to cause a 32-bp-deletion in a gene called CCR5.According to Dr. HJ this deletion would cause a nonfunctional CCR5 protein and it would be impossible to contract with AIDs (Antonio Regalado:2019). This sounds great but there are a lot of risks and concerns associated with the procedure as it has been reported that the efficiency rate using CRISPR is only 15% for the for a single gene correction (Liang *et al*., 2015). One of the major drawbacks using CRISPR editing technique is its off targets effects and it has been reported by researchers that this can cause disabilities

and unfortunately can even cause cancer in some cases (Kim *et al*., 2015; de Miguel Beriain and del Cano, 2018). This means that the doctor himself knew all the possible side effects of these experiments but did not fully make the parents aware. In short this technique is not fully safe to be experimented on reproductive cells.

First application of CRISPR in plants

In august 2012 8 brief reports on the application of CRISPR system were published (Nekrasov *et al*., 2013; Jiang *et al*., 2013), One of them included observation on transgenic rice that was intentionally mutated to increase the product and growth. However the researchers later revealed that the mutation on the particular gene was successful by using the Cas9/sgRNA system (Miao *et al*., 2013; Zhang *et al*., 2014; Wolt *et al*., 2016). The plant model species that were used with CRISPR were *Arabidopsis Heynh*., *Oryza* L., and *Nicotiana* L. (Jiang *et al*., 2013; Shan *et al*., 2013; Xie and Yang, 2013). However CRISPR gene editing in plants have shown all type of plants problem solved from herbicide resistance to industrial consumption, from increased yield to crop betterment and at the last increased resistance to both biotic and abiotic stresses (Modrzejewski *et al*., 2019).

Limitations of CRISPR and the ways in which they were improved

As we know CRISPR is under the spotlight nowadays but there are a number of factors that influence its activity. And it is important to consider these factors in order to use this technique for in vivo human gene therapy because that is a delicate procedure.

Site selection for targeted DNA

Crispr/cas9 has the ability to recognize and select 23 bp of nucleotides that contains a PAM sequence on either templates of DNA. In case of SpCas9 this PAM sequence is said to occur after every 8bp on an average estimation (Ramakrishna *et al*., 2014). This PAM motif is specific for every species, as in case of Neisseria meningitides PAM sequence is appeared to be 5′-NNNNGATT-3′ (Jiang *et al*., 2013; Ma *et al*., 2014). This feature shows a greater flexibility and higher specificity in site selection which will gradually increase with the discovery of new Cas 9 with different PAM pattern. However many reports have revealed that this is not as simple and easy as it appears to be. One report even suggests that CRISPR is not as specific as ZFNs and TALENS because it can target a shorter sequence. Hence greater the target sequence greater will be the specificity (Cradick *et al*., 2013).

Off target cleavage

Cutting outside the target sequence is caused by the sgRNA, Cas9 protein has no role in it but still if we improve the cas9 protein it can reduce these effects. The journey of improving the Cas9 system has led to mutant Cas9 systems. One of this system includes the distorting of the cas9 protein in such a manner that it only produces single stranded breaks. Then the 2 nickase enzymes (CRISPR/cas9) are used to surround the target site as one binds with the forward DNA sequence and the other with the reverse DNA sequence. In this way double stranded breaks are induced with the help of nicks. The single stranded breaks produced by off target cutting are then fused together simply by the help of DNA ligase. This mutant system when used in mammalian cells decreased off target cleavage to three folds with little to no reduction in on target efficiency (Mali *et al*., 2013; Ran *et al*., 2013; Cho *et al*., 2014). Another mutant system includes the joining of Cas9 with TALEs that have the ability to recognize and bind to any target site with improved specificity and reduced off target effects (Bolukbasi *et al*., 2015). Additionally, these mutant cas9 proteins are intentionally designed to weaken the joining of the Cas9 with target DNA strand (Kleinstiver *et al*., 2016) or the non-target DNA strand (Slaymaker *et al*., 2016) meanwhile keeping the strong on-target cleavage which is the sole purpose to reduce the unwanted DNA contacts.

Frequency of HDR

After a double stranded break, there are very less chances of a homology directed repair in mammalian cells and more chances of nonhomologous end joining, proved through an experiment in mice where Cas9 based gene editing revealed that the rate of occurrence of NHEJ associated repair was 20-60% while of HDR was only 0.5-20%, even in the presence of sister chromatids, there are more chances of NHEJ repair mechanism occurring while using CRISPR system (Maruyama *et al*., 2015). With the passage of time many new methods have been adopted to enhance the use of HDR and suppress the efficiency of NHEJ because it is more prone to errors and for that purpose small suppressor molecules of NHEJ are used (Yu *et al*., 2015) gene silencing is being done to prevent the expression of a particular gene (Chu *et al*., 2015) cell cycles are being adjusted or harmonized (Lin *et al*., 2014). These inhibitors of NHEJ are very effective as the most common; Scr7 has shown increase in rate of HDR to up to 19 folds in case of CRISPR based editing (Vartak & Raghavan, 2015). But these suppressors may have a toxic effect on host cells so to overcome this chemical suppression , alternative ways are being studied like synchronizing cell into cell cycles (late S phase and G2 phase) where HDR can occur (Lin *et al*., 2014).

Action of cas9

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Each cas9 protein from different species have different activity and a specific PAM sequence. Thus selection of a specific Cas9 is very important as it plays a great role in efficient gene editing. Such cas9 proteins are identified in a number of species including Staphylococcus aureus (SaCas9) (Ran *et al*., 2013) and *S. thermophiles* (St1Cas9) (Kleinstiver *et al*., 2015). Cas9 must be transferred to nucleus for the improved activity and for this a (NLS) nuclear location signal is attached to the Cas9 protein. This has shown improved DNA cutting activity (Shen *et al*., 2013). Another trick to increase the cleavage activity is to enhance the single guide RNA (sgRNA) concentration to Cas9 protein (Kim *et al*., 2014). But if increased outside the limit, it may become a cause of off-target cutting (Fu *et al*., 2013). The catalytic activity of Cas9 is low when we compare it to other enzymes (Jinek *et al*., 2012). But this doesn't mean that it is not useful, this feature can be beneficial for a number of things like gene suppression/activation and short term gene editing with less off target effects. But not required for other applications where catalytic activity is the sole purpose.

Some advanced CRISR systems
Keeping in view the limitations of CRISPR, scientists developed some enhanced versions of CRISPR to increase their specificity and efficiency.

CPF1 (Cas12a)
These class 2 CRISPR systems including Cas9 and Cas12a share a number of common features that to generate a double stranded break they both rely on RNA molecules (Fonfara *et al*., 2016). But Cas12a recognizes its target site with the help of a single RNA (CrRNA) molecule in comparison to Cas9 that uses CrRNA:TracrRNA. The cuts produced using CPF1 have sticky ends while that of Cas9 produces blunt ends with no overhangs (Zetsche *et al*., 2015).Lastly Cas9 has the ability to recognize PAM sequences that have more guanosine ,on the other hand Cas12a recognizes PAM sequence rich in thymine (Gao *et al*.2017). After some reports it was declared that Cas12a show less off target effects and are more specific in nature which is a major plus point in field of genome engineering (Kim *et al*.2016) Cpf1 has been successfully used for genome editing in plants rice and tobacco in the year 2016 (Endo *et al*., 2016). The simplicity and specificity of these tools make them very important to be used in base manipulation, multiplex gene targeting (Bayat *et al*., 2018) and also this has been used in clinical trials which is a major achievement (Li *et al*., 2020).

Cas9n
As described earlier there are some limitations of CRISPR-Cas9 system just like it does not have important NHEJ components , therefore when used in some bacteria it may cause death after cleavage. In such cases we use a mutant of Cas9 called Cas9 nikase (Xu *et al*., 2015). Cas9 has two sites for cleavage but if we replace one of the sites with a single amino acid it produces Cas9n which is capable of inducing a single stranded cut on the target site (Cong *et al*., 2013). These mutant tools have proved themselves in the long run due to a number of reasons, the first one being their high efficiency, they are easy to construct, are pocket friendly and does not require a lot of time in construction (Jiang *et al*., 2013;Ran *et al*., 2013). Lastly they are being used to reduce the off target effects by using a double nicking technique (Shen *et al*., 2014).

Cas13a (C2c2)
Cas13a is another achievement in the path of CRISPR editing technology. It belongs to CRISPR class 2 system and its cleavage activity depends upon two domains known as HEPN (Abudayyeh *et al*., 2016). The common feature of Cas13a and Cas12a is the ability to target and edit multiple loci due to its own single crRNA template (Abudayyeh *et al*., 2017). This tool can be utilized to control post transcriptional suppression (Elbashir *et al*., 2001). When compared with RNA interference processes Cas13a is more efficient and more specific (Abudayyeh *et al*., 2017). As a result of alternative splicing DNA is split into many isoforms which are affected when targeted with CRISPR, but with Cas13a it is possible to target and study the task of single isoforms without affecting others (Mahas *et al*., 2018). Similarly there are so many other novel and enhanced systems of CRISPR to overcome the drawbacks.

Delivery of CRISPR into the cells
This step for successful genome editing is very important as the cells to be altered must receive these CRISPR systems in order to get modified. For this purpose there are a number of methods out of which some are more successful for research work and some are more beneficial for therapeutic and clinical use.

Microinjection
This is a physical method of delivery having efficiency up to 100% (Horii *et al*., 2014). It requires a microscope and $0.5–5.0\,\mu m$ diameter glass needle which pierce the cell membrane and the material is delivered directly into the target site of the cell but while doing so it also damages the cell and it can only infect one cell at one time which makes it very time consuming and laborious (Zhang *et al*., 2008). Microinjection is best suited for in vitro and ex vivo applications.

Electroporation
It is another traditional method of delivery that uses electrical current that temporarily generates nanometer pores in the membrane to make it

permeable so that the material could enter the cell. While other delivery techniques depend upon cell type, this technique is less dependent on it. It is mostly used for in vitro work. As the procedure sometimes require high voltage current therefore it is not suitable for in vivo. Multiple cells can be edited using electroporation at one time (Kaneko *et al*., 2014). Because of its readily available sources in lab and high efficiency this technique promises a continued use for efficient delivery of CRISPR/Cas9.

Hydrodynamics

It is also a physical means of in vivo delivery, mostly enriched for liver cells (Yin *et al*., 2014). It uses a large volume of CRISPR/Cas9 editing system quickly injected into the blood stream of an animal, This rapid action causes an increase in hydrodynamic pressure due to which permeability of the cell membrane is enhanced for the time being and it allows the entry of the desired material. Mostly tail vein of the mice is preferred to be injected. The simplicity of this technique makes it a desirable method and it has proved itself a number of times including in vivo correction of Fah in mouse liver cells (Yin *et al*.2014).Then later Guan repaired hemostasis in treated mice, it is a condition in which the blood is stopped to flow out of the damaged vessel (Guan *et al*., 2016). Work on hepatitis B infected mice has also been done using hydrodynamic delivery method to stop the division and expression of the virus (Zhen *et al*., 2015). Regardless of these successful applications of this method , it is still not considered to be used in clinical works as it can cause some difficulties including stopping of the heart, enlargement of the liver and increase in blood pressure (Suda *et al*., 2007;Bonamassa *et al*., 2011). Transfection rates are low for this type of delivery and only some type more precisely liver cells are more capable of successful delivery.

Viral Vectors:

Previous physical methods discussed cannot be used in human gene therapy because they are less efficient and they are imperfect (Valsalakumarit *et al*., 2013). So we have other options of delivery that are:

Adeno-associated viruses (AAV)

As viruses can be DNA or RNA based, this one is single stranded DNA based virus that has been broadly utilized for gene therapy (Daya and Berns, 2008; Samulski and Muzyczka, 2014).These vectors are being used for multiple gene editing to study gene function in vivo (Swiech *et al*., 2015). It can infect many cells with distinctive specificities. The thing that makes AAV so special means of delivery is that they are not reported to cause any disease in human beings and also they are very efficient in their work that they do not give rise to innate or adaptive immune response or any sort of linked toxicity (Daya and Berns, 2008). AAV are so flexible that their function can be seen in in vitro, ex vivo, in vivo. It has a unique feature that the genomic material delivered through AAV can exist in two forms in the host, either directly inserted into the DNA after some modifications or outside the DNA into the cell (Deyle and Russell, 2009). The genomic material that could be packed inside the AAV particle is only 4.5-5 kb (Wu *et al*., 2010), which makes the packaging quite challenging but for now there are so many ways developed to adjust the packaging.

Lenti (LV) and adeno viruses (AdV)

LV and ADV are quite different from each other but the main reason they are being described under the same heading is that their way of delivering Cas9 system is quite similar. The major advantage of using LV or Adv delivery systems over AAV is that they have additional packaging space due to their large particle size that is 80-100nm while of AAV is only 20nm. This feature allows them to adjust and carry large insertions. These methods of delivery are being used by several groups such as for gene silencing (Voets *et al*., (2017), moreover (Kabadi *et al*., (2014) made a different combination of lentivirus/CRISPR Cas9 that has the possibility to be used for delivery in invivo. Viral vectors including LV are used to screen the function of genes more specifically they identified the genes that are important for cell death induced by West-Nile-Virus (Ma *et al*., 2015).These viral vectors have great applications in clinical studies but there are a number of obstacles in their way like they evoke immunogenicity (Follenzi *et al*., 2007; Ahi *et al*., 2011). Additionally there are chances of off target effects and cell damage (Bestor, 2000; Papapetrou and Schambach, 2016). So they must be handled with care when using for genome editing.

Non viral-vectors

There are so many non-viral vector delivery methods that are very efficient in replacing the physical and viral methods some of them are as under.

Lipid nanoparticles

They are also known as liposomes. These particles exhibit a major advantage as like other non-viral methods that they do not contain viral components, therefore they do not have safety issues or they do not give rise to immunogenicity. They can be used for ex vivo, in vivo and in vitro experiments giving a wide range of applications. While delivering CRISPR/Cas9 there are two ways of using liposomes either as delivering Cas9 and single guide RNA as genetic material which will just be like microinjection in result (Yin *et al*., 2016) or as ribonucleoprotien complexes that contains Cas9 and SgRNA which has proved successful quite a times

(Wang *et al*., 2016). In spite of all this when the nanoparticle has passed the surface of cell its translocation to the nucleus is quite a problem because in the passage it may come across the lysosomal pathway that will cause the degradation of the lysosome material which is a failure therefore this method of delivery has very less efficiency. But they can always be used after some improvement.

Gold nanoparticles

Another prospect for delivering ex vivo, in vivo and in vitro includes the use of AuNPs which when joined with Cas9:SgRNA ribonucleoprotiens make a complex that increases the efficiency rate of delivery up to 30% for causing the needed mutation (Mout *et al*., 2017), This method of delivery was also used in a mice who had Duchenne muscular dystrophy (DMD) in which the muscles are weakened due to changings in a protein called dystrophin. The gene responsible for this mutation was corrected to 5.4% after using this technique of delivery and also there was restoration of drystrophin gene expression (Lee *et al*., 2017) Now for the importance these particles are inert and the will not initiate any sort of immune response and are always a very good alternative to viral vectors.

DNA nanoclew

it is a distinctive technique for delivering CRISPR system but it special as it is not viral and only requires repeating DNA and PEI (polymer polyethylenimine), it came into existence by (Sun *et al*., 2014), A DNA nanoclew is just like a ball of yarn which is rolled on its own and joined together with the help of palindromic sequences. It has only been applied in in vitro being a new technique. When it was combined with RNP it showed 36 % efficiency in an experiment performed in 2015 (Sun *et al*., 2015).

CRISPR component delivery in plants

CRISPR-Cas9 constructs can be delivered to explants using Agrobacterium mediated gene transfer in which the vector consisting of Cas 9 protein and guide RNA is introduced into the *Agrobacterium tumefaciens* (Shan *et al*., 2018). Another method of delivery that is used is particle bombardment (Liu *et al*., 2019). Both systems have advantages and drawbacks but *Agrobacterium* infection is the most used one to obtains transgenic crops due to its simplicity and low copy number integration but it may cause cell death and browning of the plant, On the other hand particle bombardment technique is expensive which limits its use but it can infect a greater number of genotypes with in a specie as compared to *agrobacterium*

infection (Altpeter *et al*., 2016). Furthermore viral vectors have also been used to deliver CRISPR machinery into transgenic plants (Ali *et al*., 2015). Cas9 can be delivered in a plant cell in the format of DNA, mRNA or proteins. Each one of them has their own positivities and negativities. For instance DNA form is low cost and once the DNA is integrated it becomes stable. But both on target and off target rates are high of this method. If we deliver it in the form of mRNA, there are chances that the mRNA may be degraded in the way and it is also not stable after integration, but to produce Cas9 protein from mRNA is a very quick process. At the end ribonucleo-proteins can be injected directly (Woo *et al*., 2015). This method of delivery can give rise to directly the genome editing steps without wasting time. But defense mechanism may be active in eukaryotic cells after direct introduction of bacterial proteins through this method secondly the size endonuclease size is large which may become a hindrance in the pathway of introduction into the plant cell (Glass *et al*., 2018). In agricultural approaches both mRNA and nucleoprotein forms are suitable as compared to DNA form of delivery because it introduces transgenes into the plant.

Application in plants

Use of CRIPSR in Plants to combat biotic stress

Every year a great amount of crop yield is lost due to biotic and abiotic stresses. In order to fulfill our need of food we use engineered plants that have the ability to fight against bacteria, viruses, fungus, nematodes, insects and other pathogens that make up the biotic stress of a plant. There is an estimated average global yield loss of 11 to 30% just due to these pathogenic plant microbes and because of insect attack on plants (Savary *et al*., 2019) and an average 10-15% damage caused by alone viruses (Van *et al*., 2008). So since the very beginning scientists have been trying to develop new ways to introduce resistant crop varieties in the environment and for this purpose they used some conventional techniques including cross breeding, hybridization, biological and chemical mutagenesis (Langer *et al*., 2018), but there were several problems with these methods such as chances of undesired or non-targeted modifications and the process of screening required a lot of time and labour work. Conventional techniques were followed by genetically modified and genetically engineered crops which were again problematic. In last 10 years the hard work of scientists has finally been paid with the introduction of CRISPR.

Table 1A list of some examples of biotic stress pathogen that has been edited using CRISPR

Pathogen	Disease	Host	Target Gene	References
Oidium neolycopersici	powdery mildew fungal	Tomato	Deletion in MLO1 locus	Klimek-Chodacka *et al.,* (2018)
Pseudomonas syringae pv.	Bacterial disease	Tomato	SlDMR6-1 gene is edited	Wang *et al.,* (2019); Thomazella *et al.,* (2016)
*rice tungro spherical virus (*RTSV*)]*	Tungro (RNA)	Rice	Translation initiation factor 4 gamma (eIF4G) gene.	Macovei *et al* (2018)
*Turnip mosaic virus (*TuMV*)*	Viral (RNA)	Arabidopsis thaliana	eIF(iso)4E locus	Pyott *et al.,* (2016)
*Blumeria graminis f. sp. tritici (*Bgt*)*	Powdery mildew	Wheat	MLO gene	Wang *et al.,* (2014)
Erysiphe necator	Powdery mildew	Grapes	MLO7	Pessina *et al.,* (2016)
Erwinia amylovora	Bacterial fire blight	Apples	DIPM-1, DIPM-2, and DIPM-4	Malnoy *et al.,* (2016)
Pyricularia oryzae	Blast disease	Japonica rice	codons close to OsERF922	Wang *et al.,* (2016)

CRISPR has shown exceptional and promising work in developing successful biotic resistant crops in such a short period which was not possible by old techniques

CRISPR and abiotic stress in plants

Similarly this site specific genome editing technique has been successfully used in 20 crop species (Ricroch *et al.,* 2017) to either increase their yield of to make them resistance to biotic or abiotic stresses. Abiotic stress is the most serious obstacle in the development and production of agriculture. Abiotic stress involves a number of genes and mainly environmental conditions (Bhat *et al.,* 2016). These environmental factors include drought, salinity, cold and heavy metals. Drought and salinity are the major contributors in effecting the crop productivity as they affect 40 and 7% of the global area (Trenberth *et al.,* 2014). These abiotic stresses affect all the major and essential processes of a plant including photosynthesis, protein building, stomata passage and the rate at which transpiration occurs (Zhang X. *et al.,* 2014; Zhu *et al.,* 2016; Elferjani and Soolanayakanahally, 2018). Here is a list of plants that have been modified using CRISPR to make them adopt to these stress conditions.

Table 2. List of CRISPR mediated gene editing of abiotic stress genes in crops

Crop	Abiotic stress	targeted gene	References
Maize	Drought tolerance	ARGOS8	Shi *et al.,* (2017)
Corn	Drought tolerance	ARGOS8	Shi *et al.,* (2017)
Arabidopsis	enhanced Stomata responses	OST2/AHA1	Osakabe *et al.,* (2017)
Tomato	Drought Tolerance	SlMAPK3	Wang *et al.,* (2017)
Rice	salinity tolerance	OsRR22 OsNAC041	Zhang *et al.,* (2019) Bo *et al.,* (2019)
Wheat	Drought tolerance	TaDREB3 TaDREB2	Kim *et al.,* (2019)

Using CRISPR to improve plant nutrition and yield

After fighting with biotic and abiotic challenges in plants the next big challenge was to improve the crop yield to feed the ever increasing population. There is reported to be an increase in the nutrition value of sorghum and wheat under the assistance of CRISPR (Li *et al.,* 2018a; Zhang *et al.,* 2018b). In 2017 a batch of scientists worked together and they were able to mutate the promoter region of some quantitative genes of tomato under CRISPR that resulted in so many improved traits including better fruit shape and size that the non-mutated (Rodríguez-Leal *et al.,* 2017). From this study we have the idea that plants traits can be enhanced that just need a little push by utilizing CRISPR.

Table 3. Some examples of CRISPR edited crops to enhance crop yield and quality

Plant	Trait improvement	Targeted gene	References
Apple	non browning	Polyphenol oxidase	Halterman *et al*., (2015)
Mushrooms		(PPO) genes	Nishitani *et al*., (2016)
Potatoes			Waltz *et al*., (2016)
Rice	increased seed size	GW2, GW5, TGW6	Xu *et al*., (2016)
wheat	increased seed size	TaGW2	Wang *et al*., (2018)
Banana	increased quality	RAS-PDS1, RAS-PDS2	Zhang *et al*., (2018)
Rice	increased cooking Increased nutrition	Waxy gene	Kaur *et al*., (2018)
Rice	increased amylose Synthesis	SBEIIb	Zhang *et al*., (2018)
Wheat	low gluten	family α–gliadin	Sun *et al*., (2017)
Soybean	high yield Improved level of oleic acid	FAD2-1B , FAD2-1A	Sanchex-Leon *et al*., (2018)
Maize	increased level Of healthy tryptophan And lysine	PPR and RPL	Kim *et al*., (2017) Qi *et al*., (2016)

These are a very few success stories of using CRISPR and with the seen progress there are so many chances of more to come.

Industrial applications of CRISPR

As we know that biological industries require microorganism for their various processes. In order to enhance the efficiency of these microorganisms, modification was done using laborious and time taking methods. To the surprise of scientists came CRISPR as a gift to solve most of their problems with gene editing. Here is a little summary of using them to manipulate important industrial bacterial, yeast and filamentous fungi cells.

In Bacteria

E. coli is one of the well-known and significant strains used to produce many chemicals drugs and other useful biofuels in the industry. With the passage of time there is a great advancement in every field which has enabled us to increase the yield and revenue of the desired products from *E. coli* (Dellomonaco *et al*., 2010). For instance CRISPR was availed to uplift the flavonoid production (flavonoids are strong antioxidant with qualities of anti-inflammation and immunity). It was done so by finely adjusting and refining the metabolic systems of cell like TCA cycle and glycolysis. It was proved by this experiment that CRISPR can be used in this way without interfering with the cell's normal growth (Wu *et al*., 2015). In another example for the production of n-butanol, the expression of four of the cell's own gene (pta, frdA, ldhA, and adhE) was reduced using guided CRISPRi system. N-butanol is needed for the synthesis of ethanol, acetate, and succinate. These are important biofuels (Kim *et al*., 2017). Other species of bacteria were also facilitated by CRISPR for example solventogenic Clostridium.

These strains were not efficiently engineered due to some lacking like less understanding of its biological pathways and structure , secondly transformation rate was also low (Pyne *et al*., 2014; Bruder *et al*., 2016). During 2017 CRISPR engineering was done in a strain of Clostridium for Increased level of butanol. The butanol level reached to 19.0 g/L which was the highest record ever occurred through batch fermentation (Wang *et al*., 2017). Furthermore our little star was also able to produce succinate (chemical) in huge amount in *Synechococcus* elongates (Li *et al*., 2016).

In yeast and fungi

Similarly yeasts are also widely used for the production of great number beneficial products like enzymes, artificial food flavoring agents, chemicals, inexhaustible biofuels and in the production of biopharmaceuticals (Raschmanova *et al*., 2018). Yeasts have a tough body which enables them to even live in unsurvivable environments. For the production of biopharmaceuticals eukaryotic post translational modifications are very important but bacteria lacks such modifications, so yeast can be used instead (Thomas *et al*., 2013). CRISPR was applied in many yeast species including *Schizos accharomyces* pombe. The purpose of this modification was to achieve more efficient promoters. Jacob *et al* was successful in achieving a high efficiency of 98% in this process and resulted in highly efficient knockout (Jacobs *et al*., 2014). Once again fungi is used to make many pigments, organic acids polyunsaturated fatty acids and so many other things (Dufossé *et al*., 2014; Ji *et al*., 2014) But there were some problems with its usage in industry Firstly the passage of tool across the fungal cell wall was a big challenge and even if the gene editing tool was delivered, editing efficiency was low and thirdly it

was a laborious work that required a lot of time. However it was all until the discovery of CRISPR. CRISPR has been implemented successfully in Trichoderma reesei (Hao and Su, 2019) and in Aspergillus fumigatus which achieved a high efficiency in gene editing of 100% (Zhang *et al*., 2016). This once again showed the greatness of CRISPR as a power full genome editing tool.

Oncology and CRISPR

There are number of genetic and epigenetic factors involved in the cause of malignant cell formation. These factors can be tumor suppressers, cancer causing genes, chemo resistant genes and control loci. CRISPR appears to be helpful and promising in this matter because it possess the ability to correct such mutations and cure cancer (White and Khalili *et al*., 2016). Scientist are successful by using targeted CRISPR systems to generate cell lines and animal models for different type of cancers by either deleting or inserting the oncogenes or tumor suppresser genes (Platt *et al*., 2014). These animal models are also used to study the effect of specific genes in a disease (Chen *et al*., 2016). The most widely used animal model is the mice because of its exceptional qualities over other. It takes lesser time to generate mutants it is cheap to use and it is applicable in large scale mutagenesis studies (Hsu *et al*., 2014). Moreover scientists have been fruitful to generate cell lines for lung cancer (Choi and Meyerson *et al*. 2014), liver cancer (Xue *et al*., 2014) and pancreatic cancer (Chiou *et al*., 2015). CRISPR can target the oncogenes like receptor tyrosine kinase Erb2 involved in causing tumor cells (Brown and cooper *et al*., 1996). According to recent researches cas9 was employed to knockdown or repress MDR1 in osteosarcoma cells. As a result there was reduction in resistance to chemotherapeutic agents which is a good sign (Liu *et al*., 2016). In another experiment performed in vivo in mice, NANOG and NANOGP8 genes were targeted by CRISPR. These genes are take part in causing prostate cancer. However the end result was reduced tumorigenic concentration (Kawamura *et al*., 2015).

Terminating viral cells

This feature show the worth of CRISPR more as it can be implemented to destroy the replication system of virus, thus fighting with the viral diseases. It has been used to target Hepatitis B virus (HBV) (Zeisel *et al*., 2014). Researchers have been fortunate in targeting with Cas 9 both inside and outside the living organism and producing long term reduction in viral capacity and less production of antigen that is disease causing (Kennedy *et al*., 2015). Through some other tests researchers were able to knockout part of (HIV-1) from human CD4+ T-cells (Kaminski *et al*., 2016). However most of the cases are in clinical trials.

Fighting with Genetic diseases

Apart from combating with cancer, viral and bacterial infections, CRISPR are used to eliminate and target such inherited genes that causes disorders. For instance in an eye disease named as Retinitis pigmentosa in which the individual losses his sight due to the disruption of photoreceptors is successfully being targeted and mutated with RPGR gene. This gene in healthy individuals expresses protiens that are involved in vision building factors. It is a hope for all the blinded patients of Retinitis (Bassuk *et al*., 2016). There is a great room for CRISPR editing in other diseases because of its stable qualities. Other disease like cystic fibrosis that occurs a result of mutation in CFTR gene (Kerem *et al*., 1989) or sickle cell are long lasting disease which shorten the life span of the one carrying the genes (Lanzkron *et al*., 2013). With the help of CRISPR scientist were able to edit intestinal stem cells from the patient of cystic fibrosis outside its own living cell and repair their function (Schwank *et al*,. 2013). In case of sickle cell with HDR mediated Cas9 Li and his coworkers have edited the disease causing genes in pluripotent stem cells as a result no noticeable off targeting was seen (Li *et al*,. 2016). Further clinical trials are under process for sickle cell.

Conflict of interest

The authors declared absence of any type of conflict of interest for manuscript publication.

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