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CRISPR/Cas9: A Revolutionary Tool for Recent Advances in Crop Improvement: A Review

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A B S T R A C T

The recent advances in agricultural biotechnology and genetic engineering have brought numerous benefits to the food and agricultural sector by improving the essential characteristics of plant agronomic traits. Targeted genome editing using sequence specific nucleases (SSNs) provides a general method for inducing targeted deletions, insertions and precise sequence changes in a broad range of organisms and cell types. Genome editing tools, such as siRNA-mediated RNA interference, transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs) for DNA repair has been widely used for commercial purposes. However, the discovery of the CRISPR/Cas9 system as genome editing tool it has revolutionized the broad field of life sciences. Clustered regularly interspaced short palindromic repeats (CRISPR) was discovered for the first time in bacteria and archaea as a virological defensive DNA segment. CRISPR-Cas9 as an advanced molecular biological technique can produce precisely targeted modifications in any crop species. CRISPR/Cas9 owing to its efficiency, specificity and reproducibility, this system was said to be the "breakthrough" in the field of biotechnology. Apart from its application in the field of biotechnology, it is widely used in crop improvement.

Introduction

Crop plants, possess a complex genome organization and gene expression hence it is difficult or impossible to perform site-specific mutagenesis for the development of desirable agronomic trait - more indirect methods must be used, such as silencing the gene of interest by RNA interference (RNAi). But sometime gene disruption by siRNA can be variable or incomplete. The advent of genome editing, or genome editing with engineered nucleases (GEEN) or targeted genome editing (TGE) (type of genetic engineering in which DNA is inserted, replaced, or removed from a genome using artificially engineered nucleases, or "molecular scissors") through which targeted genome editing is accomplished in wide variety of agronomically important crop species using sequence specific nucleases (SSNs) (Kamburova *et al.*, 2017). The current applications of genome editing in plants, focuses on its potential for crop improvement in terms of adaptation, resilience, and end-

use. Novel breakthroughs are extending the potential of genome-edited crops and the possibilities of their commercialization (Wang *et al.,* 2015). Basically the success of genome editing relies on two natural DNA repair mechanisms they are 1) Non Homologous End Joining &2) Homology Directed Repair. Nucleases such as ZFNs, TALENs, Mega-nucleases and CRISPR/Cas can cut any targeted position in the genome and introduce modifications which are impossible using conventional RNAi. Unlike ZFNs, TALENs and mega-nucleases chimeric proteins target site recognition by CRISPR/Cas9 system is accomplished by the complementary sequence based interaction between the guide (noncoding) RNA and DNA of the target site and the guide RNA and Cas protein complex has the nuclease activity for exact cleavage of double-stranded DNA using Cas9 endonuclease (Kamburova *et al.*, 2017). In addition ZFNs, TALENs as a tools of genome editing they are very costlier as they require a protein engineering prior to use them as genome editing tool than the ideal genome editing tool called CRISPR/Cas9, which is very much cheaper and has very high efficiency in target genome editing and it is widely used in genome editing of plants in order to develop novel genotypes with desirable agronomic traits to strengthen the global food security (Fig. 1).

CRISPR/Cas9

Clustered regularly interspaced short palindromic repeats are the segments of prokaryotic DNA containing, repetitive base sequences. CRISPR plays a key role in a bacterial defense system, form the basis of a genome editing technology known as CRISPR/Cas9 that allows permanent modification of genes within organisms. CRISPRs are found in approximately 40% of sequenced bacterial genomes and 90% of sequenced archaea (Mojica *et al.,* 2005)

Major breakthroughs in CRISPR timeline

In the mid-2000sfew microbiology and bioinformatics laboratories began investigating CRISPRs (clustered regularly interspaced palindromic repeats), which had been described in 1987 by Japanese researchers as a series of short direct repeats interspaced with short sequences in the genome of Escherichia coli (Ishino *et al.,* 1987). Predictions were made about CRISPR as their possible roles in DNA repair or gene regulation (Makarova *et al.,* 2002; Guy *et al.,* 2004). A major breakthrough came in 2005 with the observation that many spacer sequences within CRISPRs derive from plasmid and viral origins (Bolotin *et al.,* 2005; Mojica *et al.,* 2005; Pourcel *et al.,* 2005). Together with the finding that CRISPR loci are transcribed (Tang *et al.,* 2002) and the observation that Cas (CRISPR-associated) genes encode proteins with putative nuclease and helicase domains (Bolotin *et al.,* 2005; Pourcel *et al.,* 2005; Jansen *et al.,* 2002;Haft *et al.,* 2005), it was proposed that CRISPR-Cas is an adaptive defense system that might use antisense RNAs as memory signatures of past invasions(Makarova *et al.* ,2006). In2007, infection experiments of the lactic acid bacterium *Streptococcus thermophilu*s with lytic phages provided the first experimental evidence of CRISPR Cas–mediated adaptive immunity (Barrangou *et al.,* 2007).

This finding led to the idea that natural CRISPR-Cas systems existing in cultured bacteria used in the dairy industry could be harnessed for immunization against phages a first successful application of CRISPRC as for biotechnological purposes (Barrangou *et al.,* 2012). In 2008, mature CRISPR RNAs (crRNAs) were shown to serve as guides in a complex with Cas proteins to interfere with virus proliferation in E. coli (9). The same year, the DNA targeting activity of the

CRISPR-Cas system was reported in the pathogen *Staphylococcus epidermidis* (Marraffini *et al.,* 2008**)**.

Natural CRISPR system

CRISPR-Cas loci comprise a CRISPR array of identical repeats intercalated with invader DNA-targeting spacers that encode the crRNA components and an operon of Cas genes encoding the Cas protein components. In natural environments, viruses can be matched to their bacterial or archaeal hosts by examining CRISPR spacers (Andersson *et al.,* 2008; Sun *et al.,* 2013). Adaptive immunity occurs in three stages i) insertion of a short sequence of the invading DNA as a spacer sequence into the CRISPR array; (ii) transcription of precursor crRNA (pre-crRNA) that undergoes maturation to generate individual crRNAs, each composed of a repeat portion and an invader targeting spacer portion; and (iii) crRNA-directed cleavage of foreign nucleic acid by Cas proteins at sites complementary to the crRNA spacer sequence.

Types of CRISPR/Cas system

Three CRISPR/Cas system types (I, II, and III) use distinct molecular mechanisms to achieve nucleic acid recognition and cleavage (Makarova *et al.,* 2011; Makarova *et al.,* 2011). The protospacer adjacent motif (PAM), a short sequence motif adjacent to the crRNA-targeted sequence on the invading DNA, plays an essential role in the stages of adaptation and interference in type I and type II systems (Deveau *et al.,* 2008; Horvath *et al.,* 2008; Mojica *et al.,* 2005; Shah *et al.,* 2013).

The type I and type III systems use a large complex of Cas proteins for crRNA-guided targeting (Brouns *et al.,* 2008; Nam *et al.,* 2012; Haurwitz *et al.,* 2010; Hatoum-Aslan *et al.,* 2011; Rouillon *et al.,* 2013; Hale *et al.,* 2009). However, the type II system requires only a single protein for RNA-guided DNA recognition and cleavage (Jinek *et al.,* 2012; Gasiunas *et al.,* 2012) a property that proved to be extremely useful for genome engineering applications (Fig. 2 and 3).

There are three types of CRISPR/Cas systems, which vary in their specific target and mechanism of action (Makarova *et al.,* 2011).

Type I systems cleave and degrade DNA,
Type II systems cleave DNA,
Type III systems cleave DNA or RNA.

CRISPR/Cas9 is a type II CRISPR/Cas system

Key components of CRISPR/Cas9

crRNA

Contains the guide RNA that locates the correction section of host DNA along with a region that binds to tracrRNA (generally in hairpin loop form) forming an active complex (Fig. 4) (Jinek *et al.,* 2014).

Tracrrna

Binds to crRNA and forms an active complex (Jinek *et al.,* 2014)

sgRNA

Single guide RNAs are a combined RNA consisting of a tracrRNA and at least one crRNA (Jinek *et al.,* 2014).

Cas9

Protein whose active form is able to modify DNA. It as different subunits like HNH domain, RuvC domain, PAM interacting domain etc. (Fig. 5) (Jinek *et al.,* 2014).

CRISPR-Cas9 as a genome editing tool

Different strategies for introducing blunt double-stranded DNA breaks into genomic loci, which become substrates for endogenous cellular DNA repair machinery that catalyze nonhomologous end joining (NHEJ) or homology-directed repair (HDR). Cas9 can function as a nickase (nCas9) when engineered to contain an inactivating mutation in either the HNH domain or RuvC domain active sites. When nCas9 is used with two sgRNAs that recognize offset target sites in DNA, a staggered double-strand break is created. Cas9 functions as an RNA-guided DNA binding protein when engineered to contain inactivating mutations in both of its active sites. This catalytically inactive or dead Cas9 (dCas9) can mediate transcriptional down-regulation or activation, particularly when fused to activator or repressor domains. In addition, dCas9 can be fused to fluorescent domains, such as green fluorescent protein (GFP), for live-cell imaging of chromosomal loci. Other dCas9 fusions, such as those including chromatin or DNA modification domains, may enable targeted epigenetic changes to genomic DNA (Fig. 6) (Doudna *et al.*, 2014).

General protocol for employing CRISPR/cas9 as a genome editing tool

Select target Genomic region

- 20 bp sequence followed by the PAM (NGG)
- Use online tools to minimize off-targeting

a) CHOPCHOP b) ZiFiT Targeter

c) CRISPRdirect

Rational design of CRISPR/Cas target.

Few tools which can help in designing the sgRNA complementary to the expected target site within target genomic location are

Select which Cas protein is to be used

Streptococcus pyogenes Cas9 (SpCas9) is the most common Cas9 for genome engineering

Different Cas proteins are used depending upon PAM sequence availability near the target genome

Design sgRNA

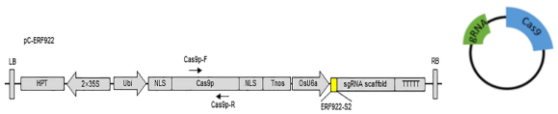
E-CRISP
Design of CRISPR constructs

CasFinder

CRISPRAnalyzeR

a) b)c)

Assembling Cas9-sgRNA construct and transferring to the desirable vector



Mobilization into the host

Lipofection
Electroporation
Agrobacterium transformation
Particle bombardment

Evaluation/Screening of target gene in the host

Sequencing
Gene Specific markers
Southern hybridization
RT PCR
Western hybridization

Potential application of CRISPR/Cas9 in agriculture

Development of viral resistant crop plant

One of the most common viral infections that notably reduces plant harvest worldwide is

caused by Geminiviruses (from the Geminiviridae family)CRISPR-Cas9 system with modified sgRNA was used to target six different regions of the bean yellow dwarf virus (BeYDV) genome in order to reduce geminivirus replication in a transgenic plant model (Baltes *et al.,* 2015). Significant reduction in copy number of BeYDVs was observed in plants that were treated with CRISPR-Cas9 utilizing four engineered sgRNAs (gBRBS +, gBM3+, gBM1−, and gB9nt+). Tashkandi *et al.,* (2018) developed a CRISPR-Cas9 system to engineer *Nicotiana benthamiana* and *Solanum lycopersicum* plants to induce immunity against tomato yellow leaf curl virus (TYLCV).

Development of disease resistant crop plants

CRISPR-Cas9 system was also evaluated for the delivery of mutations in the TaMLO-A1 and TaMLO-B1 gene of bread wheat to generate transgenic plant resistant to powdery mildew, which is a common fungal disease caused by fungi. Study was undertaken in the allotetraploid cotton genome using two sgRNAs (GhMYB25-like-sgRNA1 andsgRNA2) with high mutation frequency (Li *et al.,* 2017). The study was successful in providing plants with resistance against Verticillium wilt.

Development of abiotic stress resistant crop plants

Maize is majorly cultivated using dry farming techniques (Tykot *et al.,* 2006), and drought-tolerance in maize is a major issue. Precise CRISPR-Cas9 genome editing was carried out at the ARGOS8 locus, which is a negative regulator of ethylene response, in order to generate drought tolerant breeding (Shi *et al.,* 2017). Comparing to the wild-type, ARGOS8 variants exhibited improved grain yield under flowering and grain-filling stresses**.**CRISPR-Cas9-mediated mutation targeting ALS1 and ALS2 increased herbicide-resistance in maize (Svitashev *et al.,* 2015). ALS2 gene editing using single-stranded oligonucleotides as repair templates could successfully provide chlorsulfuron resistance to maize.

Enhancing the level of crop production

By Improving the nutritional quality of crop

Application of CRISPR-Cas9 genome editing machinery to increase amylose and resistant starch content in cereals such as rice. (Sun *et al.,* 2017) employed the CRISPR-Cas9 system to produce targeted mutagenesis in SBEI and SBEIIb genes in rice. Generated rice mutant presented amylose and resistant starch content increased by 25 and 9.8%, respectively, which, consequently, improved nutritional properties of starch in rice grain.

The seed company CortevaAgriscience (a merger of the companies Dow, Dupont and Pioneer) has taken the lead in using CRISPR-Cas technology for crop improvement. In the spring of 2016, the company's scientists developed the first commercial crop with this technology: a new generation of waxy maize. While the starch from ordinary maize kernels consists of 25% amylose and 75% amylopectin, the grains of waxy maize contain almost exclusively amylopectin (97%). Amylopectin starch is relatively easy to process and is widely used in the food processing industry and in the production of adhesives. For example, the glue on cardboard boxes and on the adhesive strips of envelopes is often derived from amylopectin starch. The problem was that the first generation of waxy maize - developed through traditional breeding - had a lower yield than traditional varieties. This has now been remedied thanks to CRISPR-Cas. The researchers at Corteva Agriscience not only

succeeded in deleting the waxy gene, they did this in most of the current elite varieties. This makes it possible to create waxy maize varieties much faster and in a way that avoids the loss of yield. These maize varieties are expected to appear on the American market in a few years, pending field trials and regulatory testing (Table 1 and 2).

Table.1 Examples of some of the crops modified through CRISPR/Cas9 (Harrison *et al.*, 2014)

CROPS	DESCRIPTION	REFERNCES
Corn	**Targeted mutagenesis**	**Liang *et al.*,2014**
Rice	**Targeted mutagenesis**	**Belhaj *et al.*, 2013**
Sorghum	**Targeted gene modification**	**Jiang *et al.*, 2013**
Sweet orange	**Targeted genome editing**	**Jia and Wang, 2014**
Tobacco	**Targeted mutagenesis**	**Belhaj *et al.*, 2013**
Wheat	**Targeted mutagenesis**	**Upadhyay *et al.*, 2013; Yanpeng *et al.*,2014**
Potato	**Targeted mutagenesis**	**Shaohui *et al.*, 2015**
Soybean	**Gene editing**	**Yupeng *et al.*, 2015**

Table.2 Advancements made in the field of agriculture and food sector through CRISPR/Cas9 (Harrison *et al.*, 2014)

GENOME EDITING TOOL	TRANSFORMATION METHOD	CROPS MODIFIED	TARGETED GENES
CRISPR/Cas9	Stable integration	*Arabidopsis thaliana*	*TT4,GAI,BRI1,JAZ1,CHLI AP1*
CRISPR/Cas9	Protoplasts; *Agrobacterium* T-DNA (Transient)	*Arabidopsis thaliana*; *Nicotianabenthamiana*	*AtPDS3,AtRACK1C,NbPDS3*
CRISPR/Cas9	Protoplasts; *Agrobacterium* T-DNA (Transient)	*Arabidopsis thaliana*; Tobacco;Sorghum; *Oryza sativa*	*OsSWEET14*
CRISPR/Cas9	Protoplasts; Bombardment	*Oryza sativa* ;*Triticumaestivum*	*OsPDS,OsBADH2,Os02g23823, OsMPK2,TaMLO*
CRISPR/Cas9	Stable integration	*Arabidopsis thaliana*	Transgene
CRISPR/Cas9	Stable integration	*Arabidopsis thaliana; Oryza sativa*	*AtBRl1,AtJAZ1,AtGAl,OsROC5, OsSPP,OsYSA*
CRISPR/Cas9	*Agrobacterium* T-DNA (Transient)	Sweet orange	*PDS*
CRISPR/Cas9	Protoplasts	*Zea mays*	*IPK*
CRISPR/Cas9	Stable integration	*Oryza sativa*	*SWEET11/13/1a/1b*
CRISPR/Cas9	Stable integration	*Oryza sativa*	*PDS,PMS3,EPSPS,DERF1,MSH1, MYB5, MYB1, ROC5, SPP, YSA*
CRISPR/Cas9	Stable integration	*Arabidopsis thaliana*	*ADH1,TT4*
CRISPR/Cas9	Protoplasts	*Arabidopsis thaliana*	*PDS3*
CRISPR/Cas9	Protoplasts; Stable integration	*Oryza sativa*	*Labdane-realated diterpenoid gene clusters on chr 2,4 and 6*

Fig.1 Types of molecular scissors

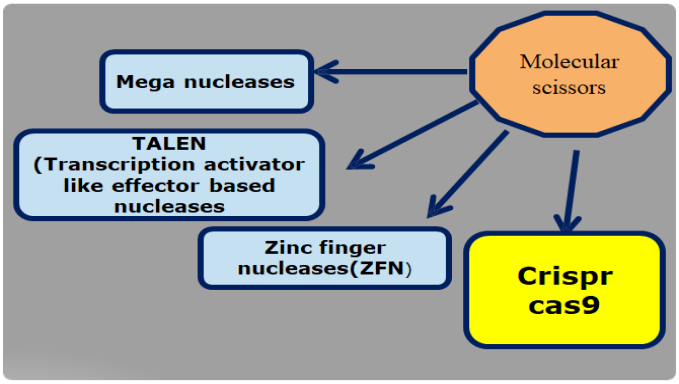


Fig.2 CRISPR timeline **(**Wang *et al.*, 2018)

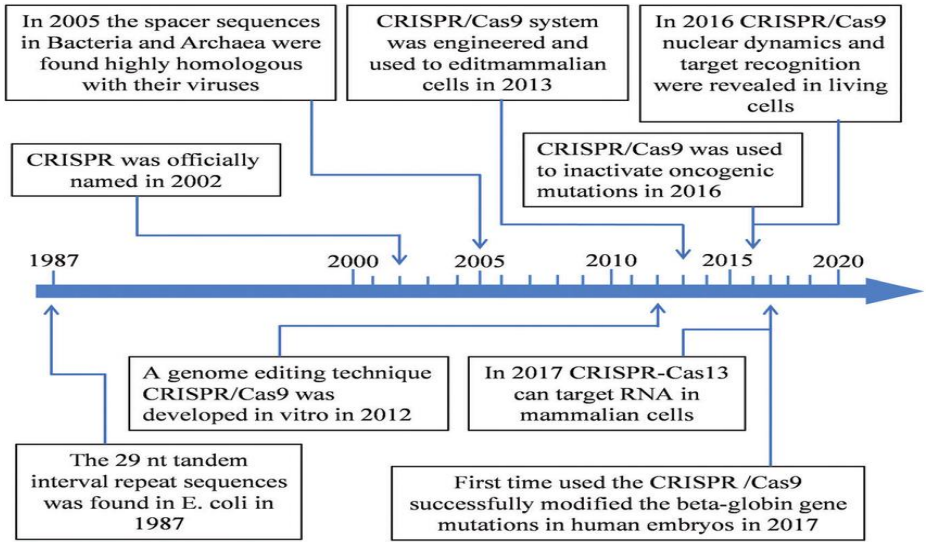


Fig.3 CRISPR/Cas9 Cascade (Zhu *et al.*, 2019)

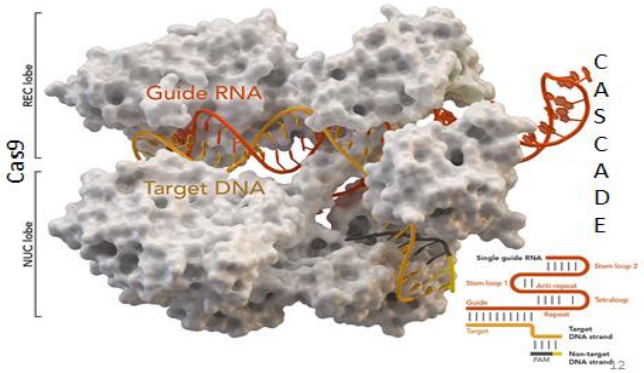


Fig.4 Genomic CRISPR Locus (Jinek *et al.*, 2014)

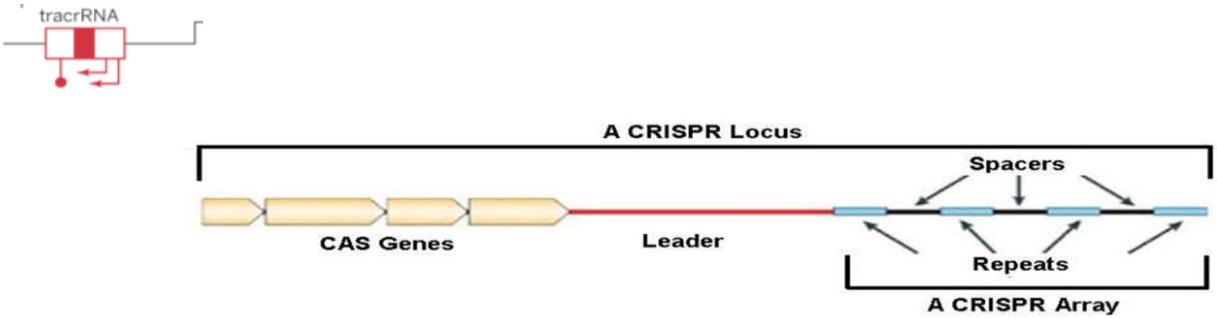


Fig.5 Subunits of Cas9 protein (Jinek *et al.*, 2014)

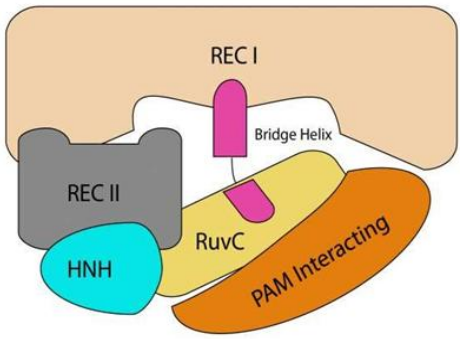


Fig.6 Working mechanism of CRISPR/Cas9 as a genome editing tool (Khatodia *et al.*, 2016)

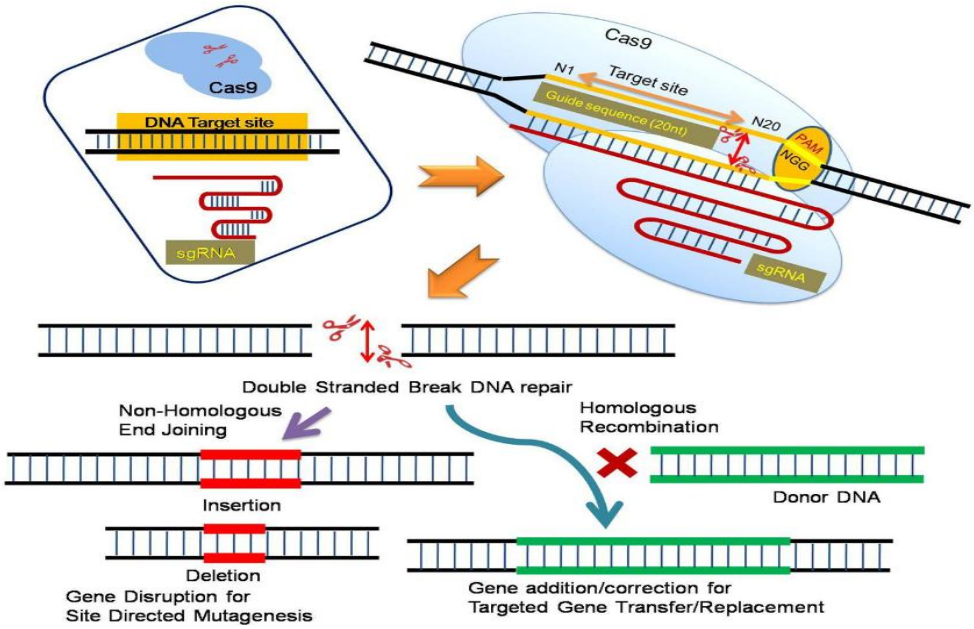


Fig.7 General protocol of CRISPR/Cas9 mediated genome editing in plants (Eş *et al.*, 2019)

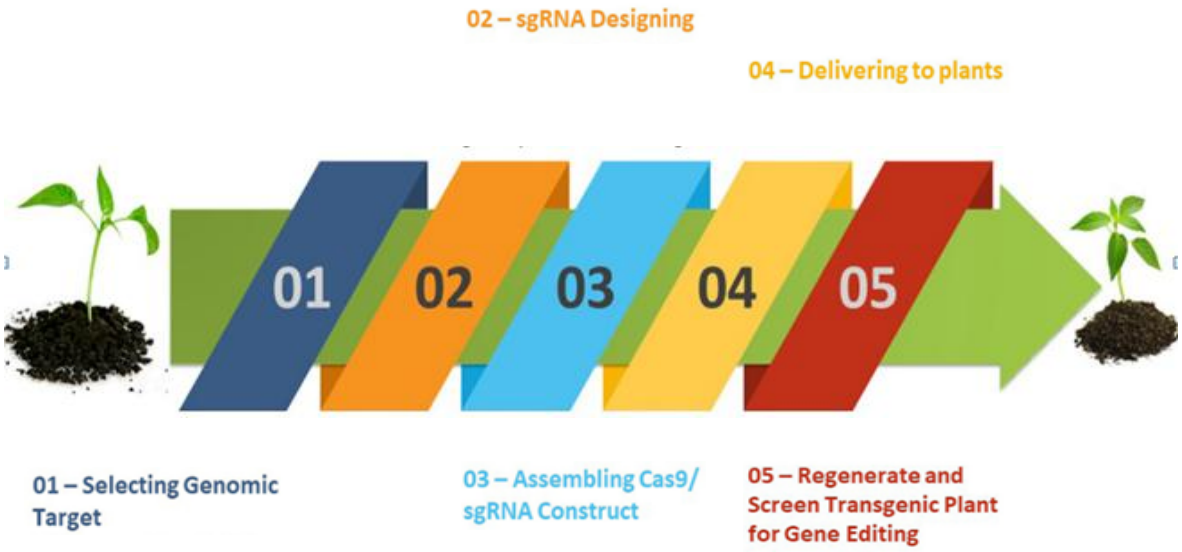
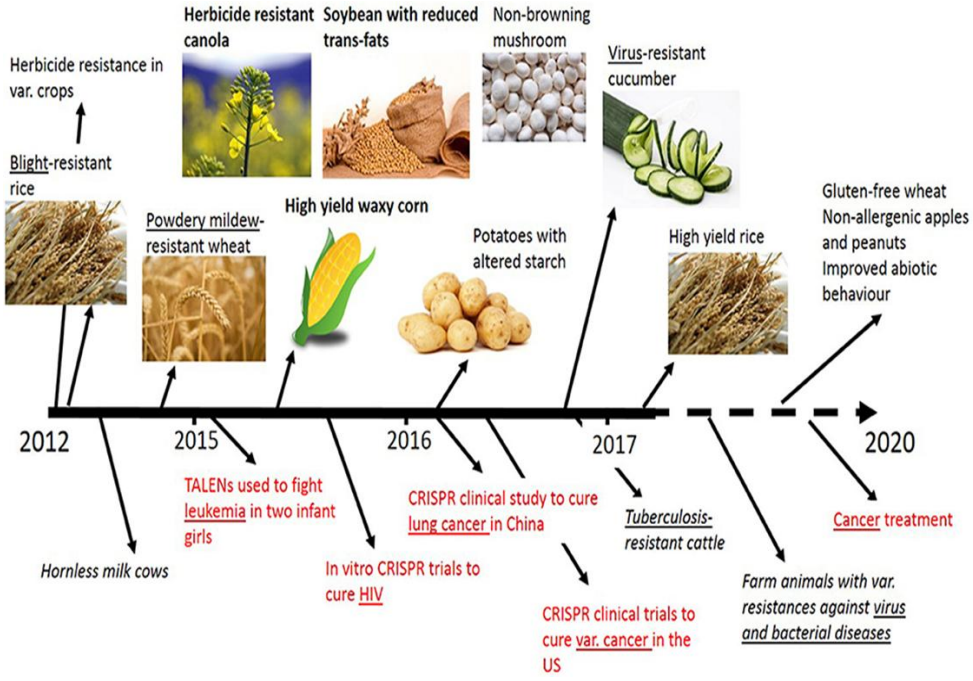


Fig.8 Chronological timeline of CRISPR/Cas9 achievements (Kamburova *et al.*,2017)



CRISPR-Cas9 technology is very important to produce potato with higher yields in a shorter time. Gene knockout of tetraploid potato (*Solanumtuberosum*) was performed by transient expression of CRISPR-Cas9 (Andersson *et al.*, 2017). RNP-delivery of CRISPR-Cas9 machinery resulted in commercial lines with higher yields without the integration of DNA.

Manipulating plant genome in order to produce bioactive compounds

Butt *et al.,* (2018) used CRISPR-Cas9 system in rice (*Oryza sativa*) in order to disrupt the carotenoid cleavage dioxygenase 7 (CCD7) gene, which modulates plant growth, reproduction, senescence, and controls an essential step in SL production. Two sgRNAs (gRNA1 and gRNA2) were engineered to target the 1st and the 7th exon and subsequently produce knockout phenotypes. Some mutants could present a significant increase in tillering. Kim *et al.,* (2016) also achieved improved lycopene and isoprene production in E. coli by manipulating the MVA pathway (mvaK1, mvaE) and ispA. Xylose production in E. coli was also significantly improved by after manipulation of the xylose pathway (xylA, xylB, tktA, talB) using the CRISPR-Cas technology (Zhu *et al.,* 2017) (Fig. 7 and 8).

Advantages

Simple design and preparation
Multiplexing genes- editing more than one gene at a time
Cheaper compare to other genome editing techniques
Possible to alter the gene expression even without altering genome-cas13 mediated gene editing

Some pitfalls/Limitations of this technology

Off target indels (insertions and deletions)
Limited choice of PAM sequences

Solutions to overcome limitations

Proper selection of gRNA
Make sure that there is no mismatch within the seed sequences (first 12 nucleotides adjacent to PAM)
Use smaller gRNA of 17 nucleotides instead of 20 nucleotides
Sequencing the crop plant before working with it
Use NHEJ inhibitor in order to boost up HDR (Homology Directed Repair)

In conclusion the genome editing is a revolutionary technology for making rapid and precise changes in the genetic material of living organisms. This can be done in the DNA of plants, microbes, animals and humans. Using this technology, scientists can change a specific DNA letter, replace a piece of DNA or switch a selected gene on or off. Over the last years; genome editing has transformed life sciences research (Genome editing was selected by Nature Methods as the 2011 Method of the Year **(**Baker *et al.,* 2011). This is mainly due to one very successful form of the technology: CRISPR-Cas9. According to the journal Science, CRISPR/Cas was the scientific breakthrough of the year in 2015.User friendly and easiness in sgRNA design makes CRISPR/Cas9 system superior over others. CRISPR/Cas9 systems use RNA for target recognition which helps this system to recognize DNA sites that cannot be recognized by ZFNs and TALENs. Genome editing with the help of CRISPR/Cas9 is highly sophisticated and reliable genome editing tools for both applied and basic plant research and breeding. Engineered nucleases can help us to modify genetics of any plant species by gene insertions or deletions or through regulation of gene expression. It is now possible to regulate metabolic pathways to get desired products with ultimate enhanced plant yield. A better understanding of mechanisms involved in response to abiotic and biotic stress along with processes involved in nutrient and water absorption will also be investigated in near future. Hence replacing of age old, time consuming traditional breeding techniques by genome editing tool like CRISPR/Cas9 direct the evolution of crops as required by mankind, and it ensures sustainability in food and agriculture sector.

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