# CRISPR-CAS9 SYSTEM: A NEW DEFINING ERA IN AGRICULTURE?

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**ABSTRAK**

*Revolusi dalam bidang rekayasa genomik terjadi sejak dikembangkannya sistem CRISPR-Cas9 yang diperoleh dari mekanisme pertahanan bakteri. Proses ini melibatkan pengikatan fragmen RNA penuntun (gRNA) yang spesifik pada DNA target yang akan menginisiasi aktivitas katalik enzim endonuklease (Cas9) pada bagian DNA tersebut. Induksi perubahan ini akan menggerakkan sistem reparasi seluler yang menghasilkan struktur genetik baru dengan karakteristik fenotipik yang diinginkan. Karena kepraktisan, fleksibiltas dan keakuratannya, teknologi ini semakin terintegrasi dalam berbagai bidang ilmu penelitian, terutama Agrikultur. Meskipun penerapan aplikasinya masih bersifat eksperimental di dalam laboratorium, hasilnya telah menunjukkan prospek yang menjanjikan di masa mendatang. Namun, banyak aspek yang masih perlu dipertimbangkan sebelum berbagai produk hasil teknologi ini di-komersilkan di pasaran. Dalam review ini, ulasan mengenai CRIPSR-Cas9 akan menjadi fokus perhatian disertai contoh penerapan mutakhirnya dalam budidaya tanaman pangan, produksi ternak dan kesehatan hewan. Kemungkinan efek negatif baik bagi manusia dan juga lingkungan dalam jangka panjang juga akan menjadi bagian dari pembahasan.*

*Kata kunci: CRISPR-Cas9, Rekayasa Genomik, Agrikultur.*

## INTRODUCTION

Ever since the knowledge and skills on genomeengineering were first discovered, it soon propelled a massive wave of interest trying to get it applied ina vast array of research, academic and therapeutic purposes. These innovative technologies assisted in shedding light on some of the most 'concealed' parts that was limited to access in analyzing at that time. It enabled the researchers to modulate both the structure and regulatory expression of genes to give a better understanding on how a certain biological system works. For instance, reconstructing a certain crop plant capable of adapting on extreme environmental pressures in order to yield higher productivity of food, fuel and/or synthetic materials.Using wide variety of animals and microorganisms as models in studying the epicenter of genetic-related diseases. In addition, developing new strategies in medical fields such as genetic repair surgery or improving the quality and quantity of a specific drug by using genetically engineered bacteria is another thing that have been done has (Hsu *et al.*, 2014). These are just some approaches of how

genome engineering can be applied to, further proving its extensive and imperative use in modern-day science.

Procedurally, the method acquire the use of certain enzymes called nucleases that will cleave specific regions of the targeted DNA thus producing what so called the Double-Strand Breaks (DSBs). In turn it will initiate cellular DNA repair mechanisms. By such process, DNA could undergo gene modifications through deletion, insertion and/or substitution in a way that would enhance or improve its genetic entities from any internal abnormalities and external hindrance. Gene correction could come in two alternative pathways: 1) homology-directed repair (HDR),applying a template homologous to the break site during either late S phase or $G_2$ phase; and 2) non-homologous end joining (NHEJ) which applied the otherwise, without any template. Engineered nuclease generally used in the field of genome editing are: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the most recent, clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) system (Orthwein et al., 2015; Gupta & Musunuru, 2014).

ZNFs contains 2 domains of protein: a DNA cleavage domain supplemented with FokI restriction enzymes and a site-specific DNA binding domain coupled with zinc-finger transcriptional factors (TFs). The cleaving domain of FokI enzymes functions as dimers of cutting scissors for a specific targeted gene recognizing both forward and reverse strand of the DNA. It was proven to be quite an efficient strategy in genome editing as it skipped several stages such as antibiotic selection and antibiotic resistance removal, in contrast to the conventional one. However, drawbacks could still have occurred, for example: the complexity of implementation making it difficult to conduct, limited target of binding sites hindering optimization of gene alteration and possible DSBs introduced to non-targeted sites (Urnov et al., 2010; Urnov et al., 2005).

Another substitutive method is TALEN displaying similar mechanism of binding and cleaving of DNA region to that of ZNFs. The differencesare that the domain only recognize a single nucleotide as opposed to triplets of it in ZNFs and with a broader DNA region target. Specificity of binding was also shown to be in higher level with less cytotoxicity when introduced into viable cells. Unfortunately, with a larger molecular size, it impairs the introduction and expression of those DNA of interest to the cells (Gupta & Musunuru, 2014; Mussolino et al., 2014).

## DISCUSSION

### CRISPR-Cas9

The latest discovery of genomic engineering-applied nuclease derived from the immune system of bacteria called CRISPR-Cas9. Currently it is considered as the most accurate, efficient and versatile genetic tool. A much more detailed discussion of this particular technology would be the center of this review.

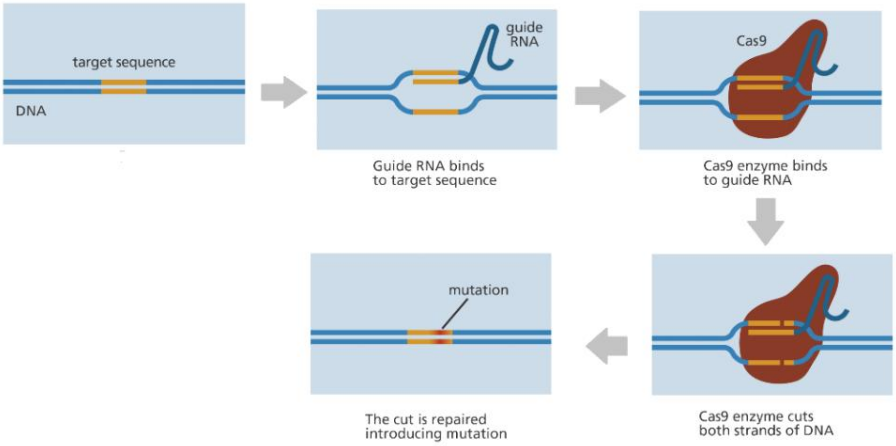


Figure 1. CRISPR-Cas9 Mechanism (https://www.yourgenome.org)

Clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) system is comprised of two compartments. The first one is an endonuclease called Cas9 serving as enzymatic DNA cutter in specific parts of the genes. The second part called guide RNA (gRNA) consist of a scaffolding RNA that will bind the targeted DNA and an RNA sequence (± 20 nucleotides) that will guide Cas9 to the gene of interest. This induced-DNA damaging will then initiate cellular DNA repair mechanism. By this strategy, scientists are able to manipulate certain genes on our genome, either removing or introducing it (Figure 1). It is considered the most advantageous technique as it is able to target many gene sites simultaneously with the most minimum non-target mutation (Jiang & Doudna, 2017; https://www.yourgenome.org).

### Application on Agriculture

The versatility of CRIPSR-Cas 9 made it widely used in many aspects of agriculture through wide varieties of organisms. One example of its application is seen in tomato plant. Researchers at Cold Spring Harbor Laboratory (CSHL) were able to alter genes that modulate phenotypic traits such as LOCULE NUMBER (fruit shape and size), FASCINATED (large fruit size), COMPOUND

INFLORESCENCE (flower proliferation) and SELF PRUNING (flowering time and growth habit). Hence, new varieties of tomatoes not commonly found in nature were able to be created being more environmental-adaptably and commercially successful (Haque *et al.*, 2018; Rodriguez-Leal *et al.*, 2017). Xu *et al.* (2016) applied CRIPSR/Cas9 tool at three genes (GW2, GW5 and TGW6) in rice that resulted in an increase grain size for up to 30%.A similar gene in wheat (TaGW6) was also knocked-out using the same technology that resulted in a much higher value of size and weight.Besides on increasing crop yields, the quality in terms of its resistance to plant diseases also has been put into research interest. Several susceptible (S) genes involving in many plant diseases have now been identified and fully-characterized. By involving the mechanism of CRISPR-Cas9 machinery, such genes could be mutated (*ex*: knocked-out, down-regulated) to produce higher resistant variants. Infectious diseases such as blast disease (caused by *Magnaportheoryzae* fungi) exemplified a major factor to significant crop yield loss in rice. By removing the S-genes (OsERF922 and OsSEC3A) to that disease, a much better defense mechanism was shown in rice. Transgenic false horn plantainshave also been successful in generating resistant cultivars to Banana Streak Virus (BSV), one of a major threat in this plants' cultivation. This was achieved by constructing CRISPR-Cas9-applied plasmid contained a mutational-silenced endogenous BSV (eBSV) and therefore, would inhibit any further protein transcriptional and/or translational of the virus when delivered to plant cells (Tripathi *et al.*, 2019). Wang *et al.* (2019) used Haploid-Inducer Mediated Genome Editing (IMGE) technology where haploid maize inducer line supplemented with CRISPR-Cas9 cassette targeting a specific gene of interest was generated in order to pollinate other inbred maize generations. The result was a homozygous double haploid (DH) maize lines carrying the desirable phenotypic characteristics. Using such approach, the requirement of multiple back-crossing could be skipped and thus making it more time-efficient. More examples of CRIPSR-Cas9 applied crop plants are shown in table 1.

Table 1. Some Examples of CRISPR-Cas9 Applied in Plant Cultivation and Breeding

| Crop | Gene(s) of interest | Role(s) of Gene(s) | Reference(s) |
|---|---|---|---|
| Banana | Endogenous banana streak virus (eBSV) genome | Banana Streak disease | Tripathi *et al.*, 2019 |
| | Phytoene desaturase (PDS) | Chlorophyll, carotenoid and gibberellin biosynthesis | Naim *et al.*, 2018 |
| Apple | | | Nishitani *et al.*, 2016 |
| Strawberry | | | Wilson *et al.*, 2019 |

| | | | |
|---|---|---|---|
| Cabbage | | | Ma et al., 2019 |
| Carrot | DcPDS, DcMYB113, F3H | Pigmentation | Xu *et al.*, 2019; Klimek-Chodacka *et al.*, 2018 |
| Cassava | eIF4E proteins (eIF4E, eIF(iso)4E- 1, eIF(iso)4E- 2, novel cap- binding protein- 1 (nCBP- 1) and nCBP- 2). | Cassava brown streak disease (CBSD) | Gomez *et al.*, 2019 |
| Eggplant | Polyphenol Oxidase enzymes (PPOs) | Fruit browning | Gianoglio *et al.*, 2018 |
| Maize | Male sterility (MS8) | Male sterile mutants (for hybrid seed production) | Chen *et al.*, 2018 |
| Potato | S-RNase (S-locus RNase) and multiple SLFs (S-locus F-box proteins) | self-incompatibility in diploid inbreeds | Enciso-Rodriguez *et al.*, 2019 |
| Tomato | Acetolactate Synthase (ALS) | chlorsulfuron-resistant | Veillet *et al.*, 2019 |
| | Nonexpressor of Pathogenesis-related gene 1 (SlNPR1) | Pathogenic-defense mechanism, drought tolerance regulation | Li R *et al.*, 2019 |
| | Tomato yellow leaf curl virus (TYLCV) genome | Tomato yellow leaf curl disease | Tashkandi *et al.*, 2018 |
| | CLV-WUS genes | Flower and fruit development, growth habit. | Haque et al., 2018; Rodriguez-Leal et al, 2017 |
| Rice | Heat-shock protein (HS) (derived from soybean) | Heat-shock response | Nandy *et al.*, 2018 |
| | OsERF922 | Rice blast resistance | Wang *et al.*, 2016 |
| Wheat | Male sterility (Ms1, MS45) | Male sterile mutants (for hybrid seed production) | Okada *et al.*, 2019; Singh *et al.*, 2018 |

Even though applications in plant cultivation seem to be more prevalent than in animal farming,however, the latter shows a promising outcome in the future. In cattle, Gao *et al.* (2017) used Chromatin Immunoprecipitation Sequencing (ChIP-seq) to identify main binding sites for inactive Cas9 (dCas9) protein located in the Bovine Fetal Fibroblast cells (BFFs). DBSs were then introduced using CRISPR-Cas9 tool to insert Natural Resistance-associated Macrophage Protein-1 (NRAMP1) gene. This gene is associated with innate defense mechanism to pathogens such as *Mycobacterium*, *Leishmania*, *Salmonella*, and *Brucella*. The newly-constructed plasmid (pSpCas9 (BB)-2A-GFP plasmid) was transferred to cells by using Somatic Cell Nuclear Transfer (SCNT) and then used for establishing transgenic cows via cloning. In chickens, immune-related genes are also found such as chTBK1 expressing the protein TANK-binding Kinase 1 (TBK1). TBK1 is involved in producing type I interferons (IFNs) as a response to pathogenic infection. The

signaling pathway of chTBK involved chSTING as mediator for IFN-β regulation. To prove this function, a chTBK1-knockout DF-1 cell line (DF-1-TBK1-C3) was createdapplying CRISPR/Cas9 machinery and transfected into chicken Embryonic Fibroblast (DF-1) cells. Results proved it was indeed required during chSTING-mediated IFN-β signaling (Cheng *et al.*, 2019). This further justifies the use of CRIPSR-Cas9 system as a versatile genetic tool for livestock genome engineering.

Table 2. Some Examples of CRISPR-Cas9 Applied in Animal Farming& Health

| Animal | Gene(s) of interest | Role of Gene(s) | Reference(s) |
|---|---|---|---|
| Cattle | Isoleucyl-tRNA synthetase (IARS) | IARS syndrome (in Japanese black cattle) | Ikeda *et al.*, 2018 |
| | NRAMP1 | Microbial defense mechanism | Gao *et al.*, 2017 |
| | POU5F1 | Early embryo development | Daigneault*et al.*, 2018 |
| | TFAM | Stress-mediated Inflammatory response | De Oliveira *et al.*, 2019 |
| Chicken | Avian Leukosis Viruses (ALVs) receptor | Avian Leukosis | Koslova *et al.*, 2018 |
| | chTBK1 | Interferon-related defense mechanism | Cheng *et al.*, 2019 |
| Dog | Canine Tumor Protein 53 (TP53) | Canine cancer | Eun*et al.*, 2019 |
| Horse | Myostatin (MSTN) | Skeletal muscle growth and development | Vichera *et al.*, 2018 |
| Japanese Rice Fish (Medaka) | | | Yeh *et al.*, 2017 |
| Sheep | | | Wu M *et al.*, 2018 |
| | ASIP | Fur coat coloration | Zhang *et al.*, 2017 |
| | BMPR-IB (FecB) | Reproductive performance | Zhang *et al.*, 2017 |
| Pig | Porcine endogenous Retroviruses (PERVs) | PERV-associated diseases | Niu *et al.*, 2017 |
| Tilapia | *vasa* | Sex determination | Li M*et al.*, 2019 |

On a different direction, animals such as Pigs has been shown to have similarities with human tissues and therefore used during xenotransplantation. One example was the silencing of the Porcine Endogenous Retrovirus (PERVs) inside pig genome applying CRISPR-Cas9 introduced in porcine primary cell line (Zhang et al., 2017). By doing so, not only that the prevention of cross-species viral transmission to human could be accomplished, but also open more doors for improving pig farming especially forproducing more resistant ones that could lower the cost for medical actions. In addition, without using too much cost in animal

feed, livestock can be modulated its genetic structure for a more favorable trait. Such approach was done in Sheep by Wu M *et al*. (2018) targeting a specific gene, Myostatin (MSTN) that is involved in skeletal muscle growth and development. By downregulating the gene activity (via CRISPR-Cas9 system) which would then be delivered to single-celled embryos, double-muscle sheep were able to be produced. These transgenic sheep would subsequently amassincreased body weight, body length and daily gain compared to the control sheep. In another research, a group of scientist tried to experiment with sheep fur colorationpattern gene called Agouti-signaling protein precursor (ASIP). By implementing CRISPR-Cas9 system, the ASIP genes was able to be altered in order to generate a wide variety of fur color in mutant sheep: 4 bp deletion at exon 2 produced badgerfaced-sheep with black fur and2 bp deletion resulted in black-white spotted fur (Zhang et al., 2017). Other examples are shown in table 2.

**Concerns**

In general, asmost genetically-modified organisms (GMOs) such as crop plants and domestic animals are, one major aspect will be considered around its safety to human health, and CRIPSR-Cas9-engineered organisms are no difference to that concern. Moreover, as the use of this technology is expected to be in an enormous amount in the future, matters concerning health issues need to be studied more thoroughly as anticipative actions. A research conducted at the National Institute of Health Science, Japan examined the possibility if these Cas9 genes being accidentally incorporated inside host cell genome during genetic editing process could indeed harm our health by analyzing the production of Cas9 proteins and their potentials as food allergens. They used a Cas9-expressed plasmid that was transfected into *Eschericiacoli* cells. Their results showed that those proteins synthetized were unstable upon heat exposure (up to 100 $^{0}$C) and were quickly degraded by Simulated Gastric Fluid (SGF). Homology comparison with known food allergen were also performed for Cas9 genes codon-optimized for human, corn and soybean which showed that no matches of allergen were found except for one in soybean which matched serine carboxypeptidase 2 in wheat (*Triticumaestivum*) (Nakajima *et al.*, 2016). These data certainly prompted the idea that if such proteins do exist in transgenic beings, further food processing methods and/ortechnologies (related to heat application) could eliminate its adverse effects and even if it is still in a slightest amount, our digestive systems (especially Gastric acid) could get rid of those proteins completely.Regardless to

that, further experimentsneed to be conducted from actual crop plants and animals genetically modified by using the CRISPR-Cas9 tool to get a more justified insight of what outcomes could possibly happen concerning food safety.

In a more recent yet shocking study, engineered transgenic cassava lines resistant to the *African cassava mosaic virus* (ACMV) targeted at the *AC2* and *AC3* genes involved in viral pathogenicity and life-cycle respectively.Their findings showed unexpected result where both test and control lines had no significant difference in terms of its viral incidence, symptoms and titres. Apparently, after further investigation, a mutated viral variant (named ACMV-AC2 H54Q) have been observed to evolved being resistant to DNA cleavage by CRISPR-Cas9. Oddly enough, sequencing displayed conserved single-nucleotide "T" insertion substituting parts in *AC2* gene resulting in a nonsense mutation and hence producing dysfunctional protein. Ironically, it somehow created an alternative Open Reading Frame (ORF)possibly encoding those altered genes (Mehta *et al.*, 2019). In recent years, scientific researches have been focusing on the technical aspects of perfecting its method in order to be as applicable and versatile as possible. But unbeknownst to us, the biological 'subjects' serving as experimental objects seemingly being ignored in their response are constantly evolving to evade such selective pressures.This certainly pinpointed a much alarming matter we should be putting as major interest in the future, and hence, to answer the question on the title above, is it really a new defining era in Agriculture? The answer is not yet. As for now, most CRISPR-Cas9-modified organisms were still experimented in laboratories and have not yet been sold commercially. If the follow-up question is, would it be in the future? The answer is, it depends. It would dependon how at the present time, every aspect circulating it have been put into thorough examination for instance as what have been described above.

## CONCLUSSION

As the world population expected to grow exponentially in the coming years, sustainingadequate food supply remains the top priority for every country. Wide range of strategiesand/or approaches have been conducted for enhancing crop and animal productivity. A prospective pathway taken applied the actions of modulating or altering gene structure and expression and therefore, minimizing the use of additional intermediary methods in agriculture such as: fertilization,

pesticidation, infection-related treatment and hybridization. To this extent, CRISPR-Cas9 genetic tool seems to provide advantageous steps in genomic engineering which previous methods failed to achieved. Its effectiveness, cost, off-target mutation and feasibility remain at the forefront compared to the others. However, it doesn't come without any limitation. With examples previously mentioned and by assuming the application of this method would be in an accelerated rate of speed and versatility, ithighlighted the need of further scientific confirmation on its safety or any side-effects that could likely occurin longer terms, not only to humans but also the environment as a whole.

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