Plant Breeding Innovation:
CRISPR-Cas9

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The CRISPR-Cas9 system is a plant breeding innovation that uses site-directed nucleases to target and modify DNA with great accuracy.[1,2]

Developed in 2012 by scientists from the University of California,Berkeley, CRISPR-Cas9 has received a lot of attention in recent years due to its range of applications, including biological research, breeding and development of agricultural crops and animals, and human health applications. These include gene silencing, DNA-free CRISPR-Cas9 gene editing, homology-directed repair (HDR), and transient gene silencing or transcriptional repression (CRISPRi).[1,3,4,5]
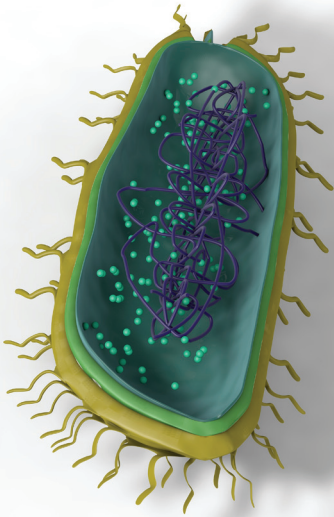
CRISPR, or Clustered Regularly Interspaced Short Palindromic Repeats, is an integral part of a bacterial defense system. It is also the basis of the CRISPR-Cas9 system.[1,2,3,5]

The CRISPR molecule is made up of short palindromic DNA sequences that are repeated along the molecule and are regularly-spaced. Between these sequences are "spacers", foreign DNA sequences from organisms that have previously attacked the bacteria.

The CRISPR molecule also includes CRISPR-associated genes, or Cas genes. These encode proteins that unwind DNA, and cut DNA, called helicases and nucleases, respectively.[1,6]

The CRISPR immune system protects the bacteria from repeated virus attacks through **THREE STEPS**:

ADAPTATION
When DNA from a virus invades the bacteria, the viral DNA is processed into short segments and is made into a new spacer between the repeats. These will serve as genetic memory of previous infections.[1,6]

PRODUCTION OF CRISPR RNA
The CRISPR sequence undergoes transcription, including spacers and Cas genes, creating a single-stranded RNA. The resulting single-stranded RNA is called CRISPR RNA, which contains copies of the invading viral DNA sequence in its spacers.[1,6]

TARGETING
The CRISPR RNAs will identify viral DNA and guide the CRISPR-associated proteins to them. The protein then cleaves and destroys the targeted viral material.[1,6]
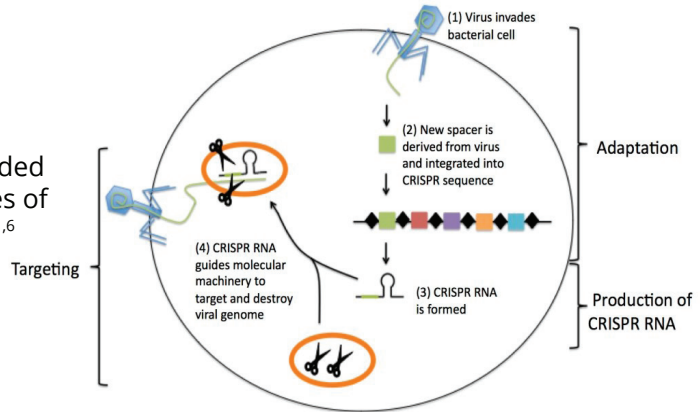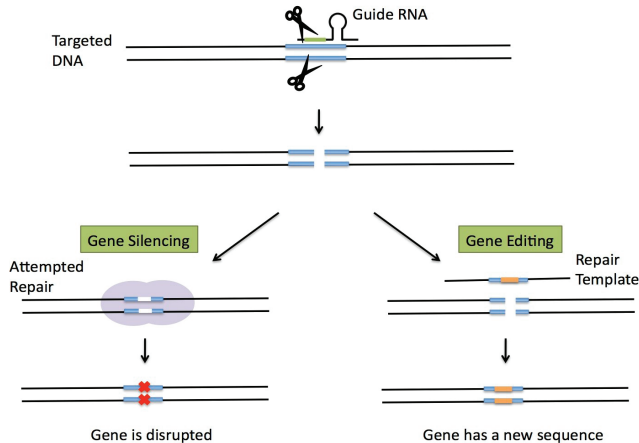

(1) Virus invades bacterial cell

(2) New spacer is derived from virus and integrated into CRISPR sequence

(3) CRISPR RNA is formed

(4) CRISPR RNA guides molecular machinery to target and destroy viral genome

Targeting

Adaptation

Production of CRISPR RNA

Photo source: Harvard University. http://sitn.hms.harvard.edu/flash/2014/crispr-a-game-changing-genetic-engineering-technique/.

Targeted DNA

Guide RNA

Gene Silencing

Attempted Repair

Gene is disrupted

Gene Editing

Repair Template

Gene has a new sequence

Scientists use the CRISPR-Cas9 systems' recognition of specific DNA sequences and apply it in the process of developing improved crops.

Instead of viral DNA as spacers, scientists design their own sequences based on their specific gene of interest. If a gene's sequence is known, it can be easily used in CRISPR. It will then act just like a spacer for the system and guide the Cas9 protein to a matching DNA sequence.[1,6]

Gene Knock-Out

Gene silencing using CRISPR starts with the use of a single guide RNA (sgRNA) to target genes and initiate a double stranded break using the Cas9 endonuclease. These breaks are then repaired by an inherent DNA repair mechanism, the non-homologous end-joining (NHEJ). However, NHEJ is error-prone and results in genomic deletions or insertions, which then translates into permanent silencing of the target gene.[4,7,8]

DNA-Free Gene Editing

CRISPR can be used for DNA-free gene editing without the use of DNA vectors, requiring only RNA or protein components. A DNA-free gene editing system can be a good choice to avoid the possibility of undesirable genetic alterations due to the plasmid DNA integrating at the cut site or random vector integrations.[4,7]

Gene Insertions or "Knock-ins"

The CRISPR-induced double-strand break can also be used to create a gene "knock-ins" by exploiting the cells' homology-directed repair. The precise insertion of a donor template can alter the coding region of a gene. Previous studies have demonstrated that single-stranded DNA can be used to create precise insertions using CRISPR-Cas9 system.[4,7,8]

Transient Gene Silencing

By modifying Cas9 so it cannot cut DNA, transient gene silencing or transcriptional repression can also be done. The modified Cas9, led by a guide RNA, targets the promoter region of a gene and reduces transcription activity and gene expression. Transient activation or upregulation of specific genes can be effectively done.[4,7]

CRISPR-Cas9 Applications

Researchers have found that CRISPR-Cas9 can be applied to nearly every organism. Early studies using CRISPR - Cas9 for gene editing have focused on crops important for agriculture. It was realized early on that the system could be used in crops to improve traits, such as yield, plant architecture, plant aesthetics, and disease tolerance.

CRISPR has been used to edit the genome of rice. The team of Ying Wang from Syngenta Biotechnology China designed several CRISPR sgRNAs and successfully deleted fragments of the *dense and erect panicle1* (*DEP1*) gene in the Indica rice line IR58025B. Improvements in yield-related traits, such as dense and erect panicles and reduced plant height, were observed in the mutant plants produced.[9]

A team of researchers from the Chinese Academy of Agricultural Sciences led by Yupeng Cai also used the CRISPR-Cas9 system to induce mutations on *GmFT2a*, an integrator in the photoperiod flowering pathway of soybean. The developed soybean plants showed late flowering, resulting in increased vegetative size. The mutation was also found to be stably inherited in the following generation.[10]

Researchers from Beijing Key Laboratory of Vegetable Germplasm Improvement, led by Shouwei Tian used CRISPR-Cas9 to target *ClPDS*, the phytoene desaturase in watermelon, to achieve the albino phenotype. All genome-edited watermelons harbored mutations in *ClPDS* and showed full or mosaic albino phenotype. This study served as a proof of concept of using the CRISPR-Cas9 system in watermelon breeding.[11]
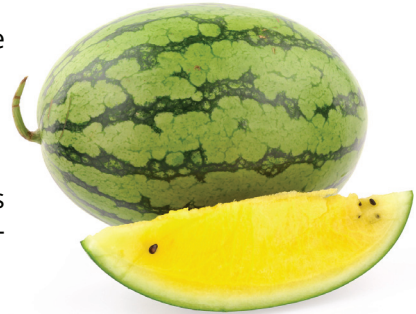
Researchers from the Chinese Academy of Agricultural Sciences and National Center for Citrus Variety Improvement and Southwest University have also developed citrus plants resistant to citrus canker caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), a serious disease of citrus, through CRISPR-Cas9.

The team targeted the promoter of the *CsLOB1* gene, which promotes canker development in citrus. The developed lines showed enhanced resistance to citrus canker compared to wild types.[12]

Cold Spring Harbor Laboratory, together with various research institutions, also used CRISPR-Cas9 to generate mutations in the flowering suppressor *SELF-PRUNING5G* (*SP5G*) in tomato to manipulate photoperiod response. The mutations brought about by CRISPR-Cas9 caused rapid flowering and enhanced the compact growth habit of field tomatoes, resulting in a quick burst of flower production and early yield.[13]

CRISPR-Cas9 has also allowed generation of animals suitable for human disease modeling. The team of Yuyu Niu from Yunnan Key Laboratory of Primate Biomedical Research applied CRISPR-Cas9 via coinjection of Cas9 mRNA and sgRNAs into one-cell-stage embryos. The team generated CRISPR-edited cynomolgus monkeys for brain disorders that cannot be fully studied in mice.[14]

Researchers from the University of California also used CRISPR for studies in gene therapy. Using CRISPR-Cas9, they corrected mutations associated with the genetic disease, β-thalassemia by creating induced pluripotent stem cells (iPSCs) from the β-thalassemia patients. The team used CRISPR-Cas9 to correct mutations in the human hemoglobin beta (HBB) in patient iPSCs, resulting in gene-corrected iPSCs with restored expression of the *HBB* gene, which can be used for gene therapy.[15]

US scientists are also studying the use of CRISPR for treating the Human Immunodeficiency Virus (HIV). They used CRISPR to edit the HIV genome out of immune cells, called T cells, from an HIV patient. Scientists found that CRISPR can prompt the HIV virus to mutate. However, more studies are still needed before CRISPR can be used to treat HIV.[6]

CRISPR has played a huge part in the increase in genome editing studies in recent years. The system has broad applications in plant and animal improvement, as well as in the medical field.

As a relatively young technique, various discoveries and innovations for its efficient use in wider applications are in the offing.

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