

CRISPR-Cas
Genome editing
in plants



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Summary

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. This is mainly due to one very successful form of the technology: CRISPR-Cas. According to the journal *Science*, CRISPR-Cas was the scientific breakthrough of the year in 2015.

The CRISPR-Cas system consists of two components: a 'guide' and a 'scissor'. Cas is the molecular scissor: it is a protein that is guided to a specific place in the genome by a CRISPR RNA molecule (guide RNA or gRNA). Once at its target, Cas cuts the DNA. This mechanism is not new in itself: bacteria have been using CRISPR-Cas for a long time to protect themselves against viruses. So rather than being invented by humans, CRISPR-Cas was devised by nature millions of years ago. People turned this system into a useful tool to perform well-targeted genome editing.

Just like other genome editing techniques, CRISPR-Cas allows scientists to make very precise changes to DNA without having to introduce foreign genes in the process. CRISPR-Cas stands out mainly because the technology is cheaper, faster, more efficient and more versatile than the alternatives. This technique is now being used in countless labs around the world. Its use has spread beyond basic research because it has proved to be a very versatile tool for gene therapy and crop improvement. Its first applications in medicine and agriculture are a fact.

This VIB dossier describes current and emerging applications of CRISPR-Cas technology in agriculture. This background file is written in an accessible way, so that anyone with a keen interest, regardless of background, will find it informative. The boxes are for those who want to learn more, but they are not essential to grasp the general idea.

Facts and figures

DNA is found in the nucleus ('core') of each cell. It carries hereditary information and holds the instructions for what the cell is and can do. The whole of the DNA in the cell is called the 'genome'. A single instruction is called a 'gene' (see Figure 1 on Page 9).

The genome is a sequence of DNA building blocks or DNA 'letters'. For example, the genome of the intestinal bacterium E. coli consists of a sequence of about 3 million DNA letters. The human genome has 3.2 billion DNA letters.

But humans don't hold the record in that field. The current genome-size champion is the canopy plant, Paris japonica, with a genome of 150 billion DNA letters.

Genes are first copied to RNA and then translated into proteins. In addition to a structural function in the cell, proteins also have a role in chemical conversions, transport of biomolecules, cellular communication and regulation.

DNA is generally stable. Nevertheless, the sequence of DNA letters can change. This is a mutation. Mutations can occur naturally in every gene, at any time and in every cell. Mutations can also be made intentionally by humans - for example by irradiating the cell or by genome editing.

The word mutation has a negative connotation. Mutations can change the function of a gene. This can be for the worse: an accumulation of mutations can lead to cancer in humans and mutations are at the basis of hereditary diseases. However, the function of a gene can also be improved by a mutation. The fact that we can digest the protein in cow's milk, for example, is the result of a mutation. Moreover, many mutations have no effect whatsoever on the function of a gene. We call these neutral mutations.

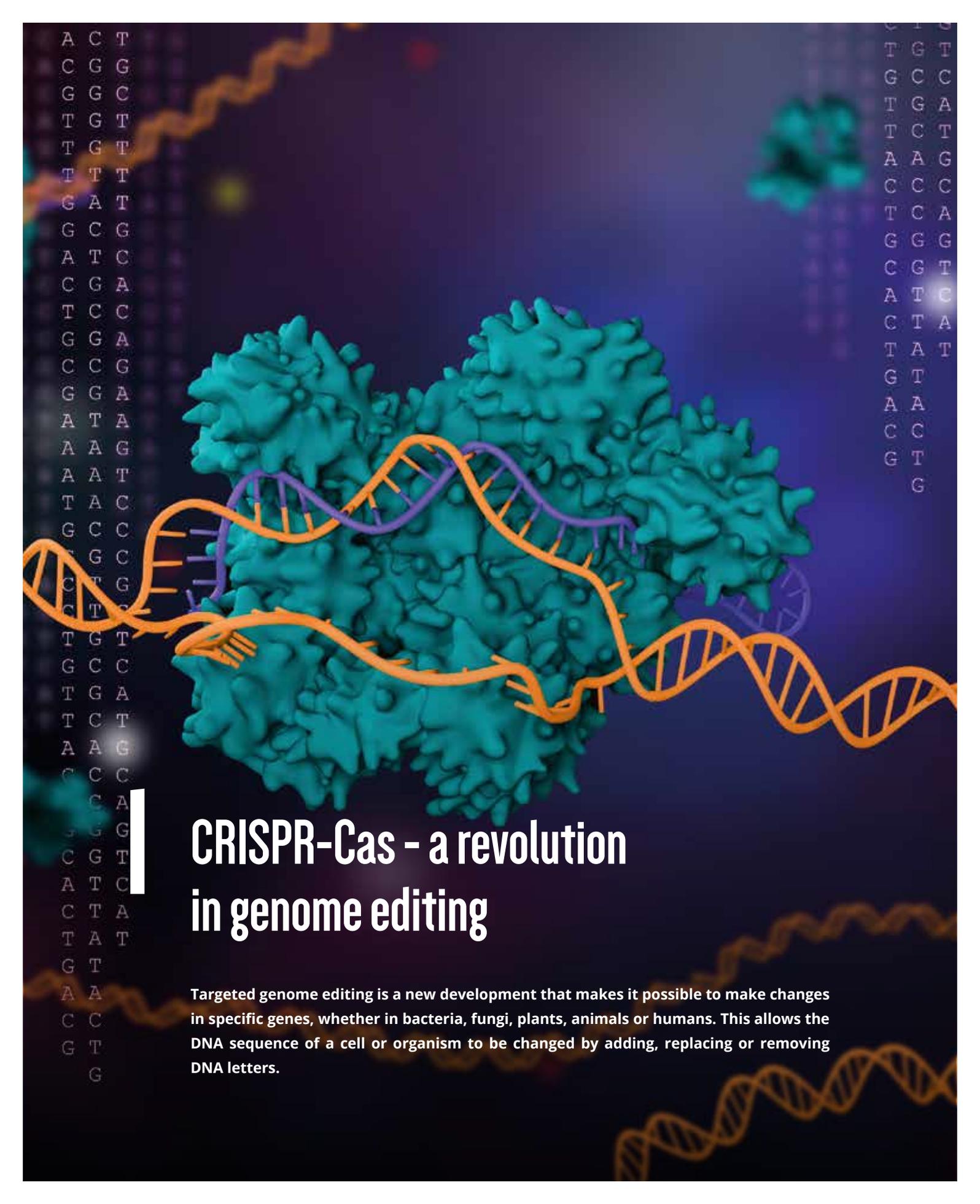
Mutations also create variation within a species. They are therefore essential for life because without mutations there would be no evolution or biodiversity. Thus, there is a delicate balance between DNA stability and evolution.

Genome editing is a way of making a specific mutation at a specific, predetermined location in the genome.

The fact that genome editing has become widespread in recent years is mainly due to one very successful form of technology: CRISPR-Cas. CRISPR-Cas makes it possible to modify DNA with unprecedented precision and efficiency.

The magazine Science called CRISPR-Cas the scientific breakthrough of 2015. The technology was developed from the CRISPR-Cas system that bacteria use to defend themselves against viruses.

In CRISPR-Cas, CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats and Cas for Caspr associated protein.



CRISPR-Cas - a revolution in genome editing

Targeted genome editing is a new development that makes it possible to make changes in specific genes, whether in bacteria, fungi, plants, animals or humans. This allows the DNA sequence of a cell or organism to be changed by adding, replacing or removing DNA letters.

Bacteria protecting themselves against viruses

The CRISPR story starts in a bacterium. The initial discovery of CRISPR sequences was reported in 1987 by Japanese scientists, who investigated the genome of the bacterium *E. coli*. They identified five identical pieces of DNA that were repeated and were separated by non-repetitive DNA sequences of identical size. At that time, these DNA repetitions were considered a curiosity since they could not be explained.

However, when scientists examined the genomes of more bacterial species, they kept seeing these same repeated DNA sequences. These species included bacteria used to make cheese and yoghurt and bacteria that naturally occur in our gut. Since then, it has been found that more than half of all bacterial species have CRISPR sequences¹.

The finding that these regular DNA repetitions always occur together with a common group of genes, CAS genes, only deepened the mystery. In **2002** a team of Dutch microbiologists decided to call the region of DNA with these repeats 'CRISPR', which is an acronym for 'clustered regularly interspaced short palindromic repeats' and called the associated genes 'CAS' genes, which is short for CRISPR-associated genes². It quickly became clear that the proteins encoded by the CAS genes function as molecular scissors that can cut DNA.

In **2005**, further research showed that the DNA sequences between the repeats are almost identical to the genetic material of viruses that infect bacteria^{3,4,5}. This type of viruses is called bacteriophages. The CRISPR region thus appeared to be a library of viral DNA fragments that the bacterium has built into its own genome. It was then suggested that CRISPR-Cas was a system for protecting

bacteria against bacteriophages. The bacterium collects DNA sequences from invading viruses and uses them, in combination with Cas proteins, to detect and cut the DNA of these attacking (see Figure 2).

In **2007**, using the yogurt-making bacterium *Streptococcus thermophilus*, scientists for the first time experimentally demonstrated that CRISPR-Cas is effectively a part of the immune system of bacteria⁶. Repeated exposure of the bacteria to a virus causes them to develop resistance over time. This resistance is accompanied by the inclusion of viral DNA fragments in the CRISPR region of the bacteria. When the scientists removed the viral sections from the CRISPR region, the resistance disappeared immediately.

Various CRISPR-Cas systems have been identified over the years and, although these systems have different characteristics, the mechanism is always the same: RNA is read from the fragments of DNA in the bacterium's CRISPR library. These pieces of CRISPR RNA then go off in search of viral genes to bind to. Next, the Cas protein, guided by the CRISPR RNA sequence, cuts the viral DNA (see Figure 2). The collection of fragments of virus DNA therefore serves as a kind of memory. This allows the bacteria to quickly recognize and fight off the virus the next time it attacks.^{7,8,9}

Researchers learn from bacteria

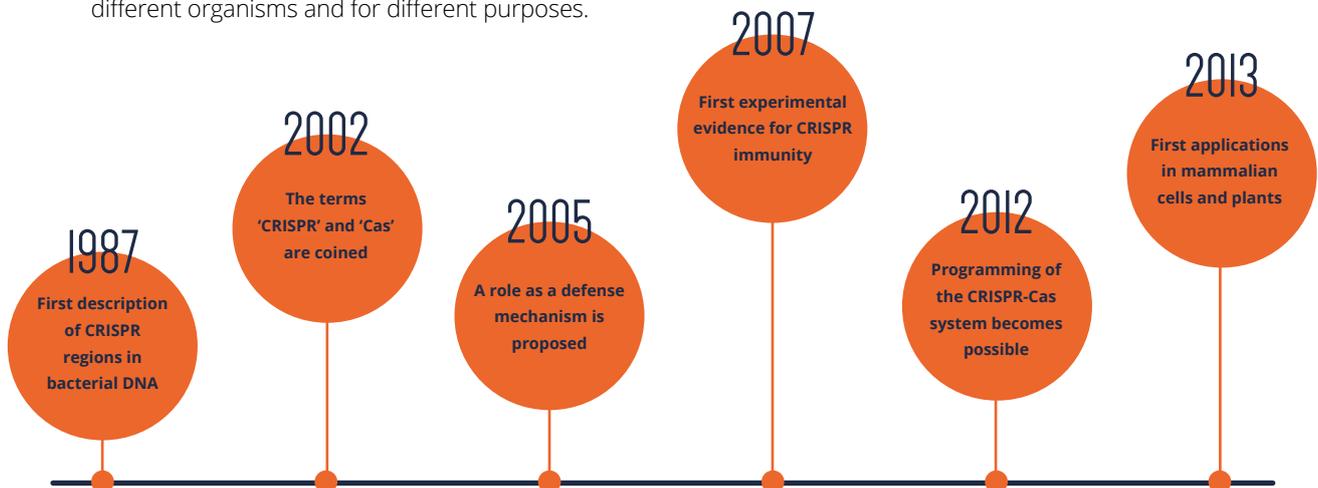
Targeted cutting ...

The great breakthrough in the use of CRISPR-Cas as a technology for editing the genomes of microbes, plants and animals came in 2012, when two independent researchers - Jennifer Doudna (UC Berkeley in the US) and Emmanuelle Charpentier (then University of Umeå in Sweden, now at the Max Planck Institute in Germany) - showed that you can reprogram the CRISPR-Cas complex. By modifying the sequence of the CRISPR RNA molecule, the complex can be made to cut at any desired location in the genome. Care must be taken to ensure that the sequence of the CRISPR RNA matches the DNA sequence where the cut is to be made^{8,9}.

Shortly thereafter, in 2013, five independent research teams, including Feng Zhang and his colleagues from the Broad Institute (MIT, USA), showed that the CRISPR-Cas system can also be used to change the DNA in human cells, mice and zebra fish^{11,12,13,14,15}. The use of CRISPR-Cas in mammalian cells was a pivotal moment in genomic editing. This was quickly followed by countless publications where the system was used in different organisms and for different purposes.

Later that year (August 2013) five research articles were published that discussed the use of CRISPR-Cas in plants^{16,17,18,19,20}. This first set of publications about its use in plants showed how immensely versatile CRISPR-Cas technology was. Plant geneticists showed that CRISPR-Cas could be used not only in *Arabidopsis thaliana* (thale cress) - a plant often used by researchers in the laboratory - but also in food crops such as rice. Later, tomatoes, wheat, maize (corn) and other crops were added to the list.

CRISPR-Cas is not the only molecular technology for editing the genome. Several techniques were developed that either use molecular scissors other than Cas9 (Cas12, previously called Cpf1, Cas13 previously called C2c2, ...) or are based on another mechanism, such as oligonucleotide-directed mutagenesis, TALEN technology, and ZFN technology. However, this goes beyond the scope of this dossier. (For an overview, see the VIB Facts Series issue 'From plant to crop: the past, present and the future of plant breeding').



THE GENOME GOVERNS THE CELL FROM THE NUCLEUS

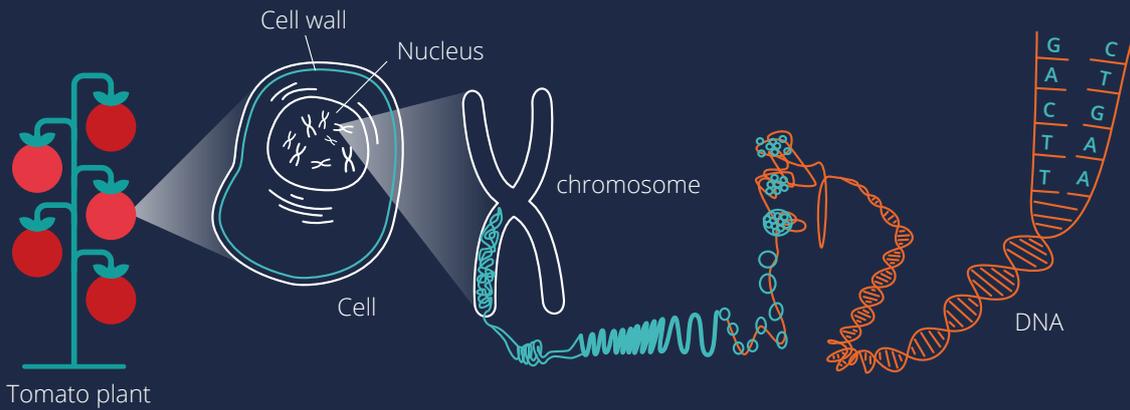


Figure 1. The genome governs the cell from the nucleus

The nucleus of each cell contains DNA, which is the carrier of hereditary information that holds the instructions for what a cell is and what it can do. The whole of the DNA in the cell is called the genome.

DNA - a double-stranded molecule in the shape of a helix - is packed into a number of chromosomes. For example, each cell of a tomato plant has two sets of 12 chromosomes or, to put it another way, two sets of 12 'packets of DNA'. If these chromosomes were to be unrolled and laid end-to-end, they would form a thread about half a meter long with a diameter of 2 nm (nanometers), or 2 millionths of a millimeter.

As soon as a cell divides, each daughter cell receives the complete genome - all the DNA packets - from the parent cell. That requires a great deal of DNA copying.

The DNA code consists of 4 'letters': A, T, C and G. The letter A on one DNA strand will always be paired with the letter T on the other strand, and vice versa. The same is true for the letters C and G. So, when we read one strand, we also know the letter order of the other - complementary - strand.

The total genome of the rice plant consists of 370 million DNA letters, while that of the potato plant has 840 million and wheat has 16 billion letters. For comparison, the human genome has 3.2 billion DNA letters. A sequence of DNA letters encoding an instruction is called a gene.

This instruction can be the recipe for a protein. In other words, the DNA code, or the gene, is read and translated into a protein via an RNA molecule. Proteins are important in forming the structural parts of the cell, but they also perform biochemical tasks. They ensure that the cell converts nutrients into energy, produces growth factors, builds a cell wall, etc. Only a small part of the plant DNA effectively codes for proteins. The rest of the DNA is important for regulating the transcription of DNA and its translation to proteins, the copying of the DNA, the maintenance of the structure of the DNA and the chromosomes, and so on.

Occasionally, an error occurs during DNA replication. This is called a mutation. An error in the code of a gene can lead to a defective protein. A mutation, however, does not need to result in a changed gene product. If mutations, whether they result in a changed gene product or not, are passed on to the daughter cells, the variation within the species increases.

... and natural pasting

Breaks in DNA are harmful. Because of this, living organisms have natural DNA repair mechanisms that detect and repair breaks. This applies just as well to the breaks caused by Cas. When Cas has cut a DNA strand, one of two natural DNA repair scenarios can occur: either non-homologous DNA end joining or homology-directed DNA repair. In practice, CRISPR-Cas technology can use both mechanisms.

In non-homologous end joining, the cell uses specific proteins to glue the two ends of the DNA break back together. However, this process is error-prone and often leads to random mutations at the site of repair, where one or a few DNA letters disappear. This can switch off the function of the gene. In many cases, however, that is exactly the intention of the researcher (see the next section).

CRISPR-CAS - USED BY BACTERIA TO FEND OFF VIRUSES

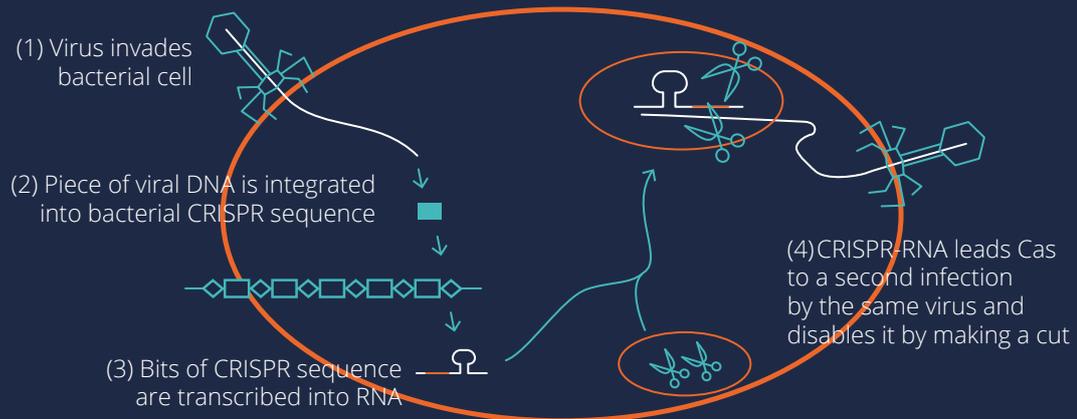


Figure 2. CRISPR-Cas - used by bacteria to fend off viruses

Viruses consist of a protein coat containing genetic material. A virus multiplies by introducing its genetic material into a cell (for example a bacterium). Next, the virus uses the hijacked cell's synthesis mechanisms to produce new viral proteins and genetic material, which are assembled into new viruses. These can, in turn, infect other cells.

Each time a bacterium is attacked by a virus (1) but survives the attack, the bacterium stores a piece of the virus DNA in its own genome, specifically in the CRISPR library (2). The bacterium translates this library into CRISPR RNA molecules (3) that guide the Cas proteins to new incoming viruses that the bacterium recognizes. Cas then cuts up the viral DNA and, in this way, repels the viral attack (4). (Figure based on reference¹⁰)

When the cell uses a new DNA sequence as template to repair the break, this is called homology-directed DNA repair or homologous recombination. For this, however, the ends of this extra piece of DNA must largely resemble the DNA sequences around the fracture. The break is then repaired by replacing the broken region with the

help of the DNA piece one provides as a template. With this, the DNA around the break is restored to its original state, or a change can be deliberately built in, depending on the template that is provided (see Figure 3).

CRISPR-CAS – USED BY PEOPLE TO ALTER DNA

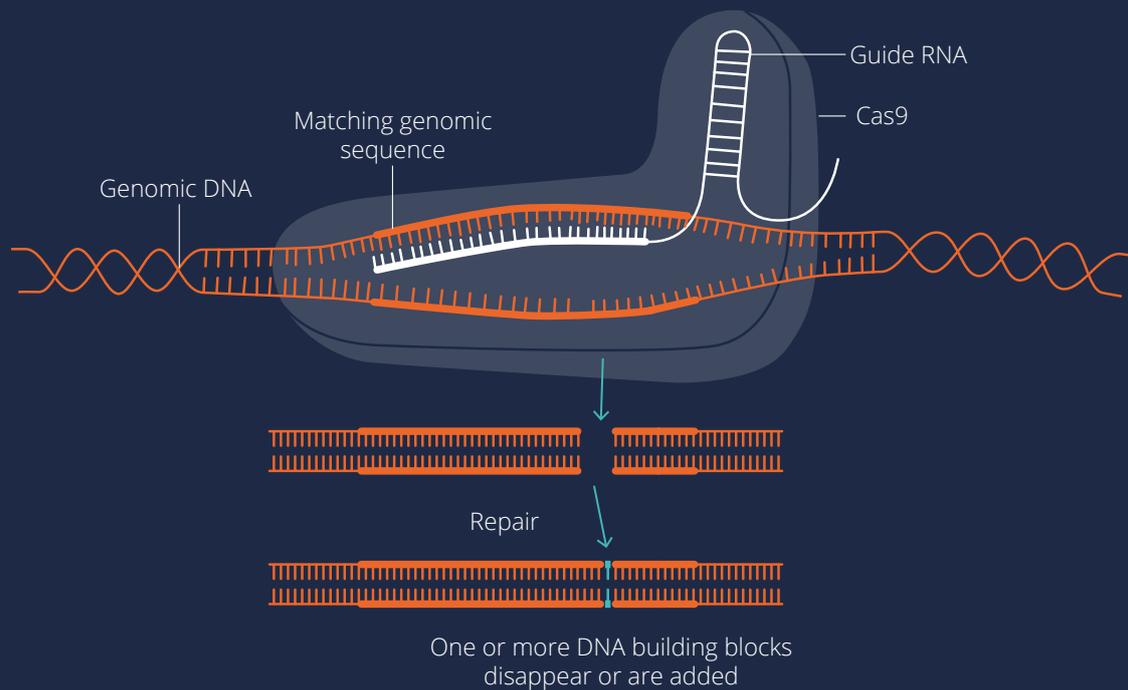


Figure 3. CRISPR-Cas genome editing

The guide RNA directs the Cas protein to a specific site on the DNA, where it causes a break (1). The cell's natural repair mechanisms will try to repair the break. During this natural process errors are often made, resulting in one or more DNA building blocks disappearing or being added, causing one or more mutations(2).

A woman with long brown hair, wearing a white lab coat with an orange collar, is working in a greenhouse. She is surrounded by lush green tomato plants with many small green tomatoes. She is looking towards the camera with a slight smile. The background shows the structure of the greenhouse.

2 CRISPR-Cas speeds-up research

Targeted genome editing is not new. Various techniques for making targeted changes to DNA exist since several years. What makes CRISPR-Cas so revolutionary is that it is very cheap, easy to use and can very precisely target specific DNA sequences. Scientists working at universities and companies therefore massively adopted CRISPR-Cas in their research.

A huge impact

Across the globe, scientists are trying to decipher molecular mechanisms and life processes in viruses, bacteria, plants, animals and humans. They want to know how living things work and how different genes, proteins and biological processes interact. This basic research provides insight into the origins of diseases such as cancer, brain and nerve diseases, cardiovascular diseases, inflammations and infections. Over time, this knowledge leads to new medicines, vaccines, diagnostic tests and treatment methods (see the VIB Facts Series 'Alzheimer' and 'Cancer').

Similar insights into growth and disease mechanisms of plants are helping scientists to raise the yields of agricultural crops, prevent damage by diseases and pests, and protect crops against extreme climate conditions such as drought.

One important element of this research is reading, deciphering and mapping of the complete genomes of organisms. Deciphering genetic codes is faster than ever thanks to the introduction of new technologies and sequencing instruments. But reading a genome is not the same as understanding it. The challenge today is to identify the genes, determine the function of the corresponding proteins, identify which other non-coding sequences are important in the genome, and so on.

With CRISPR-Cas, scientists can quickly identify the function of a gene or a particular DNA sequence. CRISPR-Cas allows them to switch off the gene of interest and see which characteristics of the cell or organism are affected. We call the resulting organisms 'knock-outs'. Multiple genes can also be studied at the same time by switching them off simultaneously.

LEGAL WRANGLING

CRISPR-Cas is the subject of a major patent conflict. Various research groups and companies claim that they made an important contribution to the discovery of CRISPR-Cas and its use as a tool for editing genomes. This has created a complex patent landscape, with contradictory arguments about ownership, infringement, and the legality of patents.

Shortly after Jennifer Doudna and Emmanuelle Charpentier showed in 2012 that CRISPR-Cas could be used to edit DNA⁹, they each applied for a patent on the technology at the American patent office (on 25 May 2013). The patent office, however, granted a patent to a competitor, Feng Zhang. He hadn't submitted his application until October 2013 but used a faster procedure. Zhang had published the first use of CRISPR-Cas in eukaryotic cells in 2013¹². Since then, even more researchers have claimed to have been the inventors, including a Lithuanian team led by Virginijus Siksnys (Vilnius University) and Luciano Marraffini from Rockefeller University (USA). In addition, hundreds of patents on the use of CRISPR-Cas for specific applications have already been submitted. This has made the patent situation surrounding CRISPR-Cas very complex.

The stakes involved in the patent dispute are high: whoever gets a patent on CRISPR-Cas can determine whether (and how much) someone must pay for using the method.

The way in which previous revolutionary breakthroughs in biotechnology - such as recombinant DNA, RNA interference and PCR - have been dealt with, could lead the way. These technologies can be used freely by academic and non-commercial research groups, while commercial companies gained access to non-exclusive licenses. This approach facilitated the broad dissemination of these techniques and could therefore also be seen to offer a solution in the CRISPR case.

The desired sequence for the CRISPR RNA molecule can be created quickly and cheaply - for example, by ordering it online from one of the countless DNA and RNA production companies. The CRISPR RNA molecules are then delivered to the laboratory by a courier service, just as you and I order shoes, clothes, books or office supplies online.

The supplied CRISPR RNA is inserted into the cells together with Cas proteins, after which a mutation is made at a location in the genome homologous to the CRISPR RNA. Next, the cells carrying a mutation are selected and grown in culture.



Basic research in plants

The emergence of this new technology to edit genomes has accelerated plant research. Since the introduction of CRISPR-Cas, countless laboratories around the world have used it to identify the functions of plant genes and to change plant characteristics. This is mainly done by causing mutations at a desired location in the plant DNA, thereby disabling a gene. After the desired DNA change is made, the researchers can allow the mutated cells to grow into complete plants. Plants, after all, have the unique property to grow a new plant from a single plant cell.

Scientists thus generate a complete genome-edited plant from one plant cell where the DNA change took place. For some plant species this regeneration happens spontaneously, in other cases the process has to be stimulated by adding plant hormones that ensure the production of roots and leaves. In horticulture, techniques such as grafting and taking cuttings have used this principle for centuries.

The first plants in which DNA changes were successfully made with CRISPR were *Arabidopsis thaliana* (thale cress), tobacco, rice and wheat^{17,18,19,20}. This was quickly followed by maize²¹ and sorghum²².

USING CRISPR-CAS TO BETTER UNDERSTAND HUMAN DISEASES

CRISPR-Cas is also widely used in biomedical research to investigate gene functions. Since the completion of the human genome project, scientists have been looking ever deeper into human cells and tissues. They systematically try to identify the functions of each of the more than 20,000 genes in our genome. CRISPR-Cas is a great addition to their toolbox as it allows a gene to be switched off easily in mammalian cells, including human cells in culture.

Scientists often work with disease models to understand diseases. These are experimental animals or cell lines in which a human disease is mimicked by means of precise genetic changes. Again CRISPR-Cas proved a valuable tool for this type of research, enabling accurate disease modeling in animals. The laboratory animals commonly used for this are fruit flies, zebra fish, mice and rats. Disease models are needed to study the onset and progression of diseases and, at a later stage, to test medicines and other interventions before they are used in people²³.



3 Applications of genome editing

The plant breeding sector is ready to embrace targeted genome editing for a variety of reasons. The technology is faster and more precise than traditional plant breeding – also compared to other genetic modification technology. Moreover, genome editing has the great advantage that breeders can easily introduce genetic variation into their crops, which is the starting point for any form of breeding. They can also do this without adding genes from other organisms - something that has fueled resistance to genetically modified (GM) crops in several countries. By using genome editing, researchers have already made disease-resistant wheat and tomatoes, drought-resistant maize, and tomatoes, soya and canola with a healthier nutrient composition.

From plant to crop

Ever since the emergence of agriculture some 10,000 years ago people have been modifying plants (see also the VIB Facts Series issue 'From plant to crop: the past, present and the future of plant breeding'). They did this by selecting the best performing plants from nature and keeping their seeds for the next sowing. In addition, crops with interesting features that arose spontaneously were selected for further breeding. This often went against natural selection, because the trait was chosen for characteristics that were convenient for humans, such as a higher yield, larger fruits or a more desirable color. The great wealth of crops that we grow and eat today is mainly due to this human selection and intervention.

Crop improvement remains essential today. Worldwide, agriculture faces major challenges: the climate is becoming more unstable; in certain agricultural regions, drought or too much rainfall are gradually making it impossible to cultivate the land efficiently. Even small temperature rises can have a major impact on the yields of certain crops.

A second challenge is the increasing world population alongside a rapid global expansion of the middle class. This increases the demand for food and fodder crops. If we want to meet this challenge, production will have to grow in step with the increasing demand.

Breeders try to anticipate these challenges by producing new varieties that have sufficient yields while being better adapted to higher temperatures, periods of drought and/or growth on soils that are less suitable for agriculture.

Finally, we must reduce the impact of agriculture on people and the environment. This can be done by fertilizing in a different way and by using pesticides more selectively. This benefits the safety and health of the farmer and the consumer, and spares, for example, useful insects.

Future plant breeding has a role in all these areas. Natural resistance mechanisms against fungi, bacteria and insects can be incorporated into our current high-yield crops. This reduces their dependence on plant protection products. Plant breeding can also be used to develop crops that use water and fertilizer more efficiently.

CRISPR-Cas as precision breeding technique

Plant breeders have a variety of methods at their disposal: from selective cross-breeding to innovative genome editing methods.

During the 20th century new plant breeding techniques based on new scientific insights and technological developments were introduced. For example, hybrids, in-vitro techniques, and marker-assisted selection have been a part of breeding practices for decades, regardless of the type of agriculture the crops are grown in.

In recent years, additional techniques have been developed that can play a role in developing new crop varieties with traits that are beneficial for the farmer, the environment, the processors, and/or the consumer. These techniques are often referred to collectively as 'new breeding technologies' (see the VIB Facts Series issue 'From plant

to crop: the past, present and the future of plant breeding'). Genome editing is the latest addition to these breeding techniques.

It is important to note the following: regardless of whether these methods have been developed recently or have existed for thousands of years, all plant breeding techniques affect the plant's DNA.

Mutations - source of genetic variation

Spontaneous mutations followed by human selection

Until the beginning of the 20th century, plant breeding was mainly an empirical selection process in which seeds or tubers from the best adapted crops were stored for the following year. This selective cross-breeding was based on spontaneous DNA mutations that occur in nature. These mutations may be due to errors that occur during the copying of DNA that takes place during cell division or may, for example, arise under the influence of radiation from the sun. However, not every change to the DNA sequence leads to new traits. In most cases mutations do not result in changes to the outward characteristics of the plant. But in certain situations, changes in a plant's DNA can result in new beneficial or detrimental characteristics. These changes contribute to genetic variation.

Our ancestors noticed these changes and selected plants with interesting new characteristics to create crops with maximum benefits for humans. The great diversity that currently exists within the cabbage family - known as 'brassicas' - is a good example of this. All brassicas (cauliflower, Brussels sprouts, kale, broccoli, etc.) are created by spontaneous mutations from the same cabbage-like ancestor. The appearance of a cauliflower, for example, is the result of one change in a single

gene²⁵. Switching off this gene in other plants also gives their flowers a cauliflower-like appearance.

Mutation-based plant breeding

The greater the genetic variation within a species, the more opportunities there are to find and combine desirable characteristics. In addition to spontaneous DNA mutations, plant breeders started to use mutation breeding in the 1930s to introduce additional variation and create new crop traits. This type of breeding uses radiation or chemicals to make changes to plant DNA at a high rate. This increases the genetic variation available for plant breeding. The result of all this irradiation is a large collection of seeds with different random DNA mutations. These seeds are then used in breeding programs to get rid of the unwanted mutations and to identify plants with desirable, improved characteristics.

Traditional mutation breeding has resulted in 3,200 improved crop varieties in more than 175 plant species, including rice, maize, wheat, banana, tomato, pumpkin and soya. The striking color of the flesh and the sweet taste of the pink grapefruit is a good example of a new crop characteristic created by this form of mutation breeding.

Crops obtained via mutation breeding have been safely cultivated and eaten for decades. Thanks to its long history of use and its role in creating improved crop varieties, mutation breeding has always and everywhere been seen as a safe and reliable way to produce crops. As a result, products developed through mutation breeding are exempted from the GMO regulations in Europe.

The disadvantage of traditional mutation breeding methods is that they easily produce thousands of DNA changes, of which only one or a few might be useful.

CRISPR WAXY MAIZE

The seed company Corteva Agriscience (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 are 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-Cas²⁴. 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.

DROUGHT-RESISTANT CRISPR MAIZE

In addition to a new variety of waxy maize, Corteva Agriscience is using CRISPR-Cas to develop a type of maize that can withstand periods of drought. To do this, Corteva Agriscience is working together with Jennifer Doudna's company, Caribou Biosciences²⁶. CRISPR-Cas was used to change the maize gene ARGOS8 in such a way that it is transcribed more often, resulting in more ARGOS8 protein in the cells. This protein is involved in the regulation of the plant stress hormone ethylene. Previous studies have shown that higher production of ARGOS8 protein leads to a better yield under stressful growth conditions such as drought.

The first field trials with the resulting maize hybrids did indeed show an increase in yield under drought stress compared to control plants, and no decrease in yield in normal conditions. Additional field trials are currently being carried out at different locations to assess its commercial potential under various conditions. It is expected that these drought-resistant maize varieties could come onto the market in 5 to 10 years.

PRECISION BREEDING IN PRACTICE

In general, the CRISPR-Cas breeding process consists of six steps. Let's take wheat as an example. The wheat varieties that we grow today are very sensitive to mildew, a fungal disease. With CRISPR-Cas, scientists have now succeeded in developing a type of wheat that is resistant to mildew.²⁹

The 6 successive steps to achieve this are:

- 1. Genome study.** A successful result of CRISPR-Cas-based breeding is always preceded by a detailed genome study. The crop characteristic that you want to modify (in this example this is sensitivity to infections with mildew) must first be analyzed in detail at the genetic and molecular level.
- 2. CRISPR design.** Once scientists have determined which DNA changes are required to increase the plant's fungal resistance, a CRISPR RNA molecule is designed. This RNA molecule determines the place where the mutation will be made.
- 3. Getting CRISPR and Cas into the plant cell.** The DNA-cleaving enzymes and the guiding CRISPR RNA molecules must be introduced into the plant cell either via *Agrobacterium* transformation, via plant viruses, or directly as a protein-RNA complex.^{30,31} In the latter two cases, no genetic material is integrated into plant DNA. After carrying out their editing task, Cas and CRISPR are spontaneously broken down by the plant cell. The final result is a plant with the desired mutation(s) in the target gene(s). This resulting plant cannot be distinguished from a plant that has acquired mutations spontaneously or via traditional mutation breeding.
- 4. Screening.** The next step is to track down the cells or plant tissue pieces in which the CRISPR-Cas system performed the desired change (or changes) correctly. This is often done by means of DNA sequencing techniques to see whether the change has been successful.
- 5. Regeneration to a complete plant.** The modified cells or plant tissue cultures are then grown to a complete plant.
- 6. Traditional cross-breeding programs.** Finally, the desired mutation is incorporated into elite varieties by traditional plant breeding methods.



Unprecedented accuracy

Genome editing techniques such as CRISPR-Cas are mainly used in plants to induce highly controlled, precise DNA mutations. Hence, we speak of 'precision breeding'. By bringing about an efficiently targeted DNA change, a gene in the plant can be switched off or on. This allows breeders to dampen unwanted characteristics or strengthen desirable ones. Such mutations can also occur spontaneously in nature.

The advantage of genome editing over traditional mutation breeding is that only desired mutations are created without additional undesirable random mutations. Various studies have shown that off-target mutations rarely occur during genome editing of plants^{27,28}. In case they do occur, they can be tracked down and crossed out. Genome editing can therefore be considered as an advanced form of mutation breeding. The result is the same, we only get there faster and more efficiently. An additional advantage is that CRISPR-Cas allows one to change different characteristics simultaneously.

Extremely versatile

CRISPR-Cas can be used in various ways as a breeding method.

Mutation-based plant breeding

CRISPR-Cas is mainly used in plants to make a small change in the existing DNA sequence, without integrating foreign DNA. It is therefore a modern form of mutation breeding that induces genetic variation in a crop quickly and specifically. The resulting crop varieties can be identical to those produced by traditional breeding methods.

Transgenesis

It is also possible to incorporate a transgene into a plant with CRISPR-Cas. A transgene is a

DNA fragment from a different species. This second application is very similar to crop breeding by traditional GM technology. There is, however, an important difference: with CRISPR-Cas, the researcher can control precisely where the new gene is inserted in the plant DNA. With traditional GM technology scientists have little control over where the transgene will be integrated (see Chapter 4 'The difference between genome editing and genetic modification'). In some cases, the location of the newly-integrated gene in the genome can affect the plant's existing characteristics.

To avoid any unintended side effects due to random transgene insertion in GM crops, different versions of the modified crop are made instead of just one. These versions differ in the genomic location where the extra genes are inserted into the plant's genetic material. This was also the case with the development of, Golden Rice - rice that is genetically modified to produce provitamin A. The initially chosen version failed to produce an optimal yield because the inserted provitamin A genes disrupted the action of an important growth gene. This meant that a different version had to be used. This delayed the development of Golden Rice by several years (see the VIB Facts Series 'Golden Rice').

These complications are easily avoided by using genome editing methods such as CRISPR-Cas. In addition, CRISPR-Cas can be used to introduce different transgenes at the same location in the genome so that they are passed from the parent to the daughter plant as a single unit.



MILDEW-RESISTANT WHEAT

The development of wheat that is resistant to the fungal disease mildew is a good example of the power of genome editing technology. Today, farmers use fungicides to combat mildew. In this mildew-resistant wheat, the genes responsible for sensitivity to mildew have been disabled so that pesticide use can be greatly reduced²⁹.

Wheat's sensitivity to mildew is determined by its MLO gene. This gene codes for a protein that the fungus exploits to invade plant cells. In other words, MLO proteins form a weak point in wheat's defense against mildew. Disabling this gene is therefore an attractive way to make the plant resistant. However, the difficulty lies in the size and complexity of the wheat genome. Bread wheat, for example, has six copies of each gene. To make wheat resistant to mildew, all six copies of the MLO gene must therefore be switched off. By using radiation or chemicals (traditional mutation breeding, see earlier) this is simply not feasible as this technique does not target specific genes. Chinese researchers accepted the challenge to produce mildew resistant wheat by using genome editing and succeeded in switching off all six MLO genes in the plant.

The American biotechnology company Calyxt plans to develop this wheat commercially. Currently, trials are being conducted in test fields to see whether the crop trait is robust under open air conditions. At the same time, the fungal resistance trait is being crossed into various wheat varieties via traditional breeding methods. If all goes well, these wheat varieties could be sold to farmers by 2022.



MAKING GRAPEFRUIT RESISTANT TO CITRUS CANKER

*The cultivation of citrus fruit involves many challenges. One of these is citrus canker, which is caused by the bacterium *Xanthomonas citri*. The most effective way to combat the disease is to grow resistant varieties. However, breeding citrus trees by traditional methods is a challenging and lengthy process.*

*Researchers at the University of Florida (USA) have succeeded in using CRISPR-Cas to turn off the *CsLOB1* gene in grapefruit plants. The bacterium *Xanthomonas citri* exploits the encoding protein to colonize the plant. Grapefruit varieties with a disabled *CsLOB1* gene manage to ward off this bacterial infection, which makes them resistant to citrus canker³².*

FROM CRISPR BANANAS TO PEANUTS TO SOYA AND SUGAR BEET ... AND WHATEVER ELSE IS IN THE PIPELINE

Universities and companies are working on many more crops to obtain useful varieties: wheat with reduced gluten levels; hypoallergenic peanuts; disease-resistant bananas and sugar beets; mildew-resistant grapes and tomatoes; soya and canola with a healthier fatty acid composition; and tomatoes with five times more of the antioxidant lycopene than wild varieties.

A smiling man with a beard, wearing a straw hat and a plaid shirt, is holding a green tomato in a field. The background is a lush green field of tomato plants.

4 The difference between genome editing and genetic modification

Just as with GM technology, genome editing is used to purposefully modify one or a few crop characteristics. However, the similarity stops there.

No foreign DNA

Genetic modification of a plant allows new genetic information to be incorporated into its DNA. The new DNA fragment may come from a crossable species or from a species that the crop cannot cross with. The products of this method are called genetically modified crops or GM crops. Over the years, various GM crops have been developed by companies and public research institutions. Since 1996, GM varieties of various crops have been grown worldwide, both on a large industrial scale and a small, local scale, especially in North and South America and in Asia³³.

Compared with traditional genetic modification, genome editing is simpler, more efficient and targeted. What's more, it leaves no trace behind. It is possible to turn genes on or off with CRISPR-Cas without inserting foreign DNA into the plant. The CRISPR-Cas components can be introduced

into the cell as an RNA-protein complex. After the components have done their work and made the desired DNA change, they are simply broken down in the plant cell. Because CRISPR-Cas does not incorporate any new DNA fragments, it is also described as 'footprint-free' DNA modification.

Another approach is to introduce the genes encoding the CRISPR-Cas system into the plant via *Agrobacterium* transformation. In this case, foreign genes are indeed integrated into the plant DNA. Nevertheless, a final product that does not contain any additional, foreign genes can be obtained by outcrossing the integrated CRISPR-Cas genes in a traditional cross-breeding program. Again, no trace of the genome editing is left behind, apart from the desired mutation.

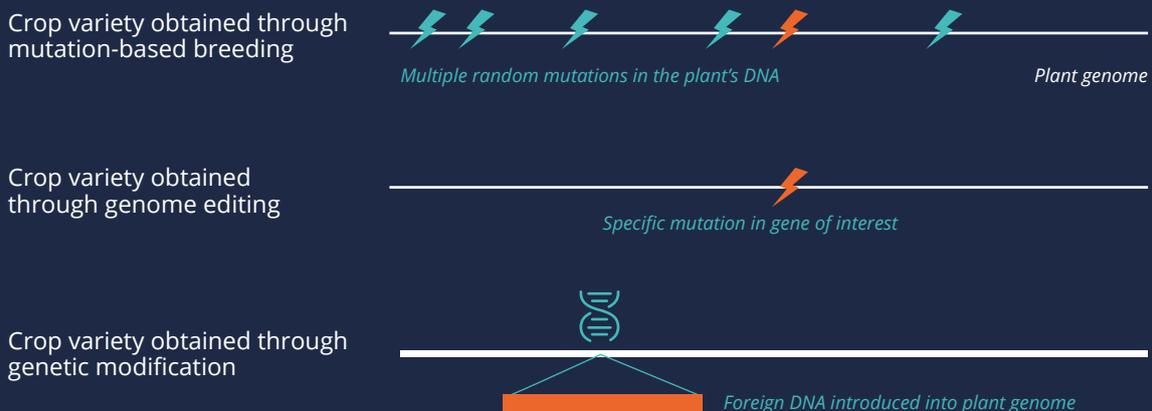


Figure 4. Illustration of the differences at DNA level between mutation-based breeding, genome editing, and genetic modification

No selection markers

In traditional genetic modification, a DNA construct - a sequence of different genes - is introduced into the plant's genome. This construct contains selection marker genes in addition to the gene of interest. Selection markers make it possible to easily select plant cells that have integrated the gene of interest.

Frequently used selection markers include genes for antibiotic resistance, herbicide tolerance or fluorescence. It is a major advance that precision breeding with CRISPR does not require selection markers. By reading the DNA sequences of plant cells on a large scale, biotechnologists can now work without the need for markers.

CRISPR-Cas thus makes it possible to change a crop characteristic in a highly targeted way, without the insertion of selection markers, foreign genes, or other genomic scars.

European regulations

European regulations, however, do not recognize this distinction. In the context of plant breeding, Europe has two types of regulations. A first one for putting new plant varieties on the market and a second specific regulation for GM crops. Since 2008 there has been a debate on whether the products of new breeding techniques, such as CRISPR-Cas, fall under the European rules for GM crops.

In July 2018, the European Court of Justice ruled that agricultural crops in which mutations have been made with CRISPR-Cas must be regarded as GM crops.³⁴ They must comply with the extremely strict conditions of the European directive for

genetically modified organisms. Other countries - outside Europe - have opted not to put these agricultural crops under such stringent legislation (see the 'Other countries, other choices' box).

Scientists and researchers received the court's ruling in disbelief. They do not understand why radiation-derived mutants do not fall under these rules, but the CRISPR-Cas mutants do. CRISPR-Cas mutants are, after all, at least as safe, if not more, as well as much more cost-effective.

Due to this ruling a great deal of much-needed precision breeding will be halted - in Europe at least. Twenty years of experience with the legislation on GM crops in Europe has shown that market authorization for the cultivation of these crops is systematically blocked by the EU, even when the European Food Safety Authority (EFSA) has evaluated the crop positively.

The ruling also creates a problem in the enforcement of the legislation. There are no foolproof detection methods that can demonstrate the difference between the genetic changes made using conventional breeding techniques and those made by genome editing. It is almost impossible to check the market access of imported CRISPR-Cas crops. However, Europe can still turn the tide by bringing its regulations into line with international practice.

OTHER COUNTRIES, OTHER CHOICES

The US government makes radically different choices when it comes to crops that have been improved by CRISPR-Cas technology. The US Department of Agriculture decided that the new waxy maize (see page 19) does not require regulatory authorization because the crop does not meet the department's criteria for a genetically modified organism. The department has also allowed the Calyxt mildew-resistant wheat (see page 22) to be commercialized without having to undergo the GM crop regulation process³⁵.

The worldwide success of CRISPR-Cas technology in agriculture will to a large extent depend on the position of local governments. In addition to the US, Brazil, Argentina, Chile, Japan and Israel also assess the products of genome editing on a case-by-case basis and CRISPR-Cas-processed crops are not automatically classed as GM crops. On the contrary, if the crop contains genetic variations that may equally well have been obtained by crossing or through random mutations, they conclude that the crop is non-GMO.

In November 2018, at the request of a group of eight countries, the Committee on Sanitary and Phytosanitary (SPS) Measures of the World Trade Organization (WTO) issued a memorandum on genome editing. The memorandum states that the new instruments for genome editing can significantly reduce the costs and deadlines for generating new crops, thereby making public researchers and technology companies better able to support local needs and challenges. This applies in particular to developing countries. The memorandum calls for a globally harmonized approach to genome editing. An approach based on sound scientific knowledge. The WTO members that have supported this initiative so far are Argentina, Australia, Brazil, Canada, Colombia, the Dominican Republic, Guatemala, Honduras, Jordan, Paraguay, the US, Uruguay and Vietnam³⁶.

5 Conclusion

Genetics has developed at an enormous rate: in less than one human lifetime we have gone from the discovery of the double helix structure of DNA (1953) by James Watson, Francis Crick, and Rosalind Franklin to genetic modification with restriction enzymes and PCR in the 1980s, large-scale genome analysis since 2000, and now the development of genome editing.

Future basic research using CRISPR-Cas will focus on, amongst other things, the development of new methods for the efficient and safe introduction of Cas proteins and their guiding CRISPR RNAs into cells and tissues of complex organisms. Today, rapid advances in this technology already allow us to make unprecedentedly accurate changes in the DNA of almost all living things.

In addition, many new applications are beginning to emerge in healthcare as well as in agriculture. New crop varieties generated in laboratories are now ready for field trials or are on the verge of market launch. In healthcare, we have never been closer to a successful implementation of gene therapy as we are today. All this thanks to the new 'toolkit' for genome editing that is CRISPR-Cas.

But the regulatory framework is still far from clear and seems, at least in Europe, to be going the wrong way. This is a challenge for policy and regulatory authorities. Technologies and their products evolve rapidly and must be continuously monitored and regulated. The effects they have on the environment and their risks to human and animal health must be kept to a minimum. Despite this, regulation should not paralyze innovation and block development of useful products. Government policy should be proportionate and non-discriminatory.

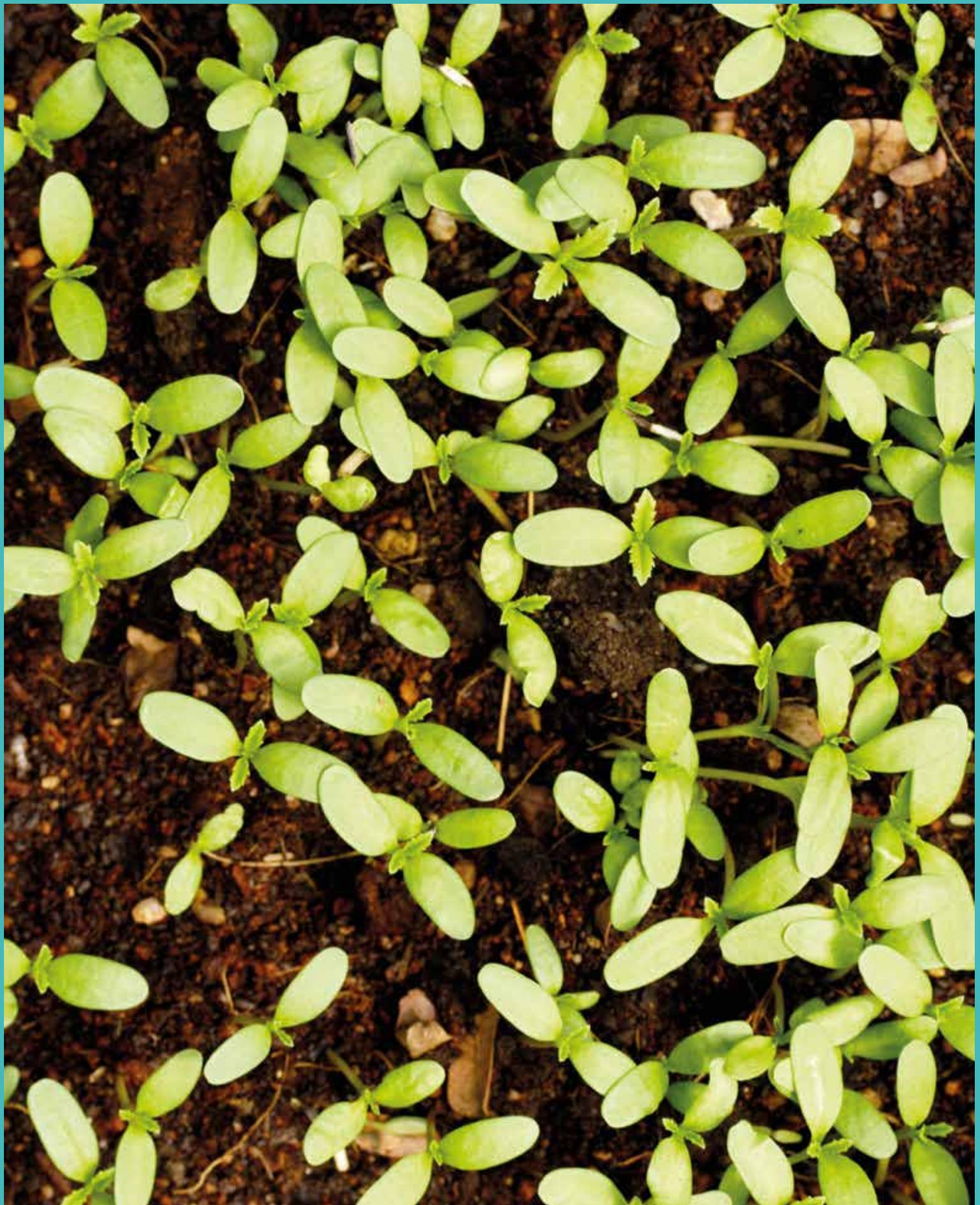
In addition, a dialog with the end user - in this case the consumer - is important. Two-way communication also requires scientists and industry to listen to the concerns and arguments of the consumers. Not only the what, how, and why should be discussed, but above all we have to get together to discuss and think about which direction we want to take in agriculture.

The price and convenience of the new CRISPR-Cas technology allows for democratic use. Let this technology become available to as many different plant breeders as possible - breeders who work with a wide range of crops. Let us help them to enter into dialog with their stakeholders and those who use their products.

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