Background: CRISPR/Cas (description of the technology)

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) system is used in the laboratory to make targeted changes to the genome of an organism (genome editing). The method is currently being intensively developed and is used mainly in plant and animal breeding, medical research and basic research. This background paper describes the natural function of CRISPR/Cas in bacteria and then explains how CRISPR/Cas is used as a molecular biological technique to cut DNA at specific regions in the genome of target organisms. It provides, in particular, a detailed explanation of the processes with which the CRISPR/Cas gene scissors can be introduced into plant cells and how changes can be made to the genome.

Origin of CRISPR/Cas in bacteria

In bacteria, naturally occurring CRISPR/Cas systems serve as an immune defense against invading viruses [1]. CRISPR/Cas helps bacteria to "remember" previous viral infections and is an intrinsic part of a defense strategy in the event of reinfection with the respective virus. Pieces from the genome of the virus are integrated into the genome of the bacteria in this process, which enables the bacteria to recognize and cut the genome of the virus if re-infection occurs [2; 3]. Researchers examined this bacterial defense system in detail and adapted it for molecular biological applications in the laboratory [4; 5]. The CRISPR/Cas system was subsequently detached from its original function in bacteria and adapted for applications in human, animal and plant cells.

CRISPR/Cas systems exist naturally in many different genera of bacteria [6]. Scientists are currently using these different CRISPR/Cas systems to further develop the gene scissors as a molecular tool to access and change more genomic regions of target organisms than previously possible. The most commonly used gene scissors are CRISPR/Cas9, in which the cutting component Cas9 comes from the bacterium *Streptococcus pyogenes*. However, other CRISPR/Cas systems are also being used to expand the application possibilities of the gene scissors, e.g. CRISPR/Cpf1 or CRISPR/Cas13 to modify RNA.

CRISPR/Cas as a molecular biological technique

Genome editing includes site-directed nucleases (SDN) and oligonucleotide-directed mutagenesis (ODM). The site-directed nucleases encompass the CRISPR/Cas gene scissors,

TALENs, zinc finger nucleases and meganucleases. They are used to cut DNA at specific regions in the genome of target organisms.
The focus of this background information is on the CRISPR/Cas gene scissors since this is currently the most frequently used genome editing technique in plants [7; 8]. The other SDNs work on similar principles but are not discussed further here.

Process of a CRISPR/Cas application in plants

A CRISPR/Cas application in plants is typically a multi-step process:

1. First of all the CRISPR/Cas experiment has to be planned and the various components designed and assembled.
2. Plant cells or tissues are usually used in a cell culture for the application of CRISPR/Cas.
3. The gene scissors are generally introduced into the plant cells in the form of DNA and are formed ("expressed") there.
4. When the gene scissors are inside the cell nucleus, the target sequence is recognized and cut. Different genome-edited cells arise.
5. New plants are regenerated from the genome-edited cells using cell tissue culture techniques.
6. The changes at the target sequence are examined and suitable plants selected by the user. The DNA for the formation of the gene scissors still present in the plant genome is removed by further crossings.

The following text describes the different steps in more detail.

1. Design of the experiment

The experiment starts at the computer. This is where many decisions have to be made about the experiment. The CRISPR/Cas gene scissors consist of a detection and cutting component. With the help of the recognition component, the gene scissors find the target sequence on the genome. The gene scissors cut the DNA there and can thus induce a change at the target site.
First of all, the recognition component of the gene scissors, also called guide RNA or gRNA, has to be designed [9]. This is an important step that has to be well considered because it can already reduce unwanted changes in the genome [10; 11] (see background information on the risks of CRISPR/Cas). How well this works depends, among other things, on the computer program used, the settings in the program and the experience of the user. The exact DNA-sequence of the genome of the plant to be modified must be known in order to design suitable gRNAs in the target gene. The guide RNA is then synthesized by biotechnology companies and inserted into the plant cells together with the gene scissors.

2. Work in cell culture

The components of the gene scissors are introduced into the plant cells or plant tissue in so-called cell cultures in the laboratory [12]. Cell cultures enable scientists to cultivate plant cells outside the plant in a culture medium. For this purpose, parts of organs or pieces of tissue (also called callus) are removed from the plants and transferred to a suitable nutritional medium. Plant cells have an impermeable cell wall, which must first be removed in order to make the cell membrane below accessible for certain techniques. Plant cells without a cell wall are called protoplasts. Plant cells are cultivated in nutritional media with numerous substances for further growth and further use. The production and cultivation of protoplasts (so called protoplast technology) is currently only established for a limited number of crops.

3. Introducing the gene scissors into plant cells

The CRISPR/Cas system can be introduced into the cell in different forms. One possibility is to introduce the DNA that carries the genetic information to form the gene scissors (see Figure 1) [13]. The actual gene scissors are subsequently formed from this DNA. However, the gene scissors can also be introduced directly into the cells as an enzyme complex that is produced in the laboratory [12; 14; 15].

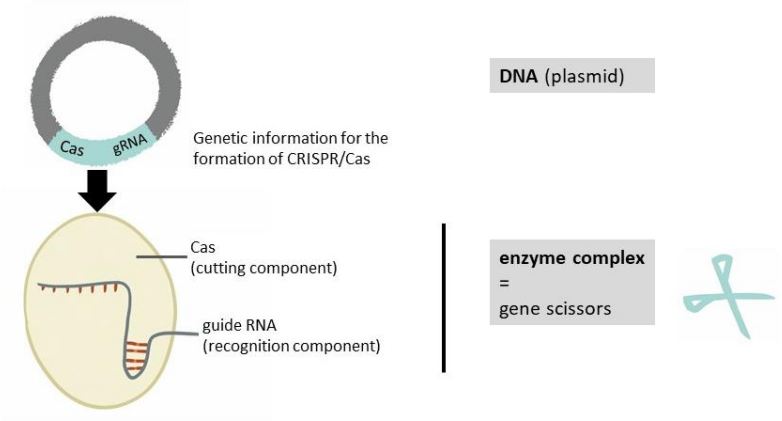

Figure 1: CRISPR/Cas can be introduced in various forms into plant cells.
The gene scissors can be introduced in different forms. One possibility is to introduce the DNA that carries the genetic information to generate the gene scissors. This circular DNA is also called a plasmid. The actual gene scissors are formed from this DNA. The gene scissors themselves are an enzyme complex consisting of a cutting component Cas and a recognition component called guide RNA that is used to identify a specific DNA sequence of the target organism. The guide RNA guides the gene scissors to the part in the genome where it is supposed to cut. The gene scissors can also be introduced directly into the cells as a ready formed enzyme complex.

There are various techniques that can be used to introduce the gene scissors into plant cells. The choice of a suitable technique depends both on the respective plant species and on the form of the gene scissors. Either the DNA carrying the information to generate the gene scissors or the already formed gene scissors are introduced to the cell nucleus of the plants.

The DNA to generate the gene scissors is mainly introduced using old genetic engineering techniques. These techniques have been used for over 30 years to introduce DNA into plant cells. The integration of the DNA into the genome of the host cell is a random process, which can often lead to unwanted changes in the genome (more information in the background information on the risks of CRISPR/Cas).

The DNA to form the gene scissors is introduced primarily with the help of the soil bacterium *Agrobacterium tumefaciens*, thus using the old genetic engineering. For this purpose, pieces of tissue (e.g. pieces of leaf or flowers) are, for example, soaked in a solution with the agrobacterium. The agrobacterium is then used as a transport vector and transfers the DNA carrying information to generate the gene scissors into the plant cells.
Another way of introducing the DNA is particle bombardment of the plant cells with very small metal particles (e.g. gold) using a gene gun. The particles are coated with the DNA for the formation of the gene scissors and are shot into the plant cells at high pressure. The DNA is released from the particles in the cell nucleus when it is hit. However, particle bombardment can also hit other parts of the cell, for example, so-called chloroplasts. Chloroplasts are responsible for photosynthesis in the cell and have their own DNA, which can be unintentionally inserted into the genome (for more information see background information on the risks of CRISPR/Cas). The DNA can also be introduced with the aid of a chemical, i.e. PEG.

The ready formed gene scissors (i.e. enzyme complex), synthesized in the laboratory from the DNA for the formation of the gene scissors, can be introduced into plant cells either by particle bombardment with a gene gun or with PEG. Both methods require protoplasts, i.e. cells without a cell wall. Their subsequent regeneration into whole plants remains challenging and has not been established for many crops, which still limits such applications [15].

Once the respective forms of the gene scissors (i.e. either in form of DNA or as an enzyme complex) are in the cell, there are different ways in which CRISPR/Cas is activated.

In case the DNA to form the gene scissors is introduced into plant cells, it can be incorporated into the genome of the cell and passed on to other cells (Figure 2). The result is a transgenic organism that carries the genetic information for the formation of the gene scissors. In the next step, the gene scissors are formed and can then recognize and cut the target sequence. In the last step, the integrated DNA to form the gene scissors is removed by further crossings so that only the changes at the target sequence remain in the genome. However, it is also possible that unintended changes in the genome arise and persist (see background information on the risks of CRISPR/Cas). The integration of DNA carrying the genetic information for the gene scissors into the genome of the plants is currently the most common way of introducing genetic scissors into plant cells.

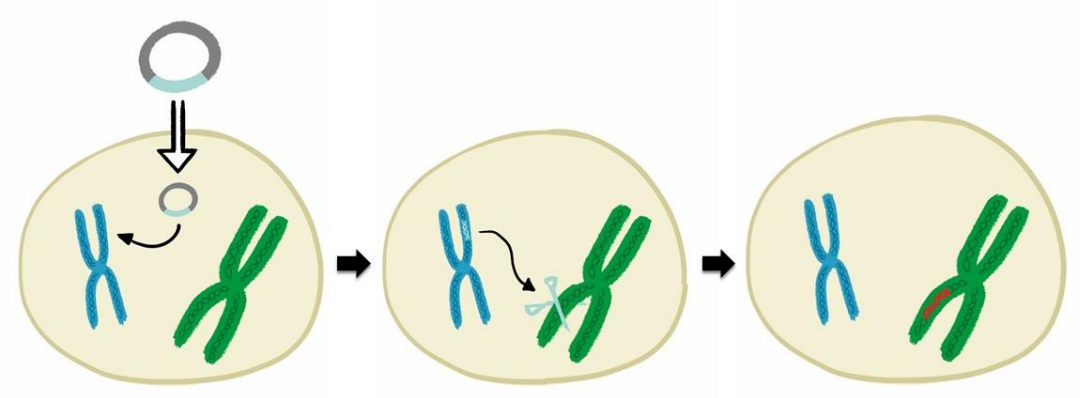

Figure 2: Integration of the DNA to form the gene scissors into the genome.
The DNA to form the gene scissors is incorporated into the chromosome shown here in blue. The result is a transgenic organism that carries the genetic information for the formation of the gene scissors. The gene scissors are then formed. They recognize and cut the target sequence on the chromosome that is shown in green. In the last step, the integrated DNA is removed and only the changes at the target sequence are supposed to remain in the genome.

If CRISPR/Cas is introduced into the cells as an enzyme complex, it can act directly in the cell, recognize the target sequence and cut at this site (Figure 3). The enzyme complex is degraded in the cell after a certain period of time and is then no longer present in the cell [16; 17]. This does not generate an intermediate transgenic organism. Nevertheless, it can cause unintended changes in the genome of the plant that must be considered (see background information on the risks of CRISPR/Cas). The efficiency of the introduced, ready formed enzyme complex is much lower than when the DNA to form gene scissors is integrated into the genome of the target plant, and depends on many different factors [15]. This is why the DNA technique is still the predominant CRISPR/Cas application in plants [18].

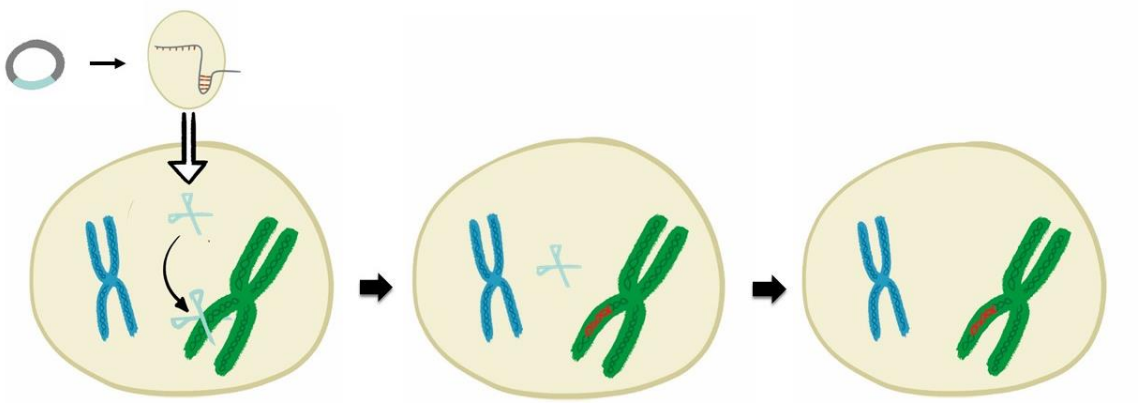

Figure 3: The gene scissors are introduced as an enzyme complex.
With this option, the gene scissors are introduced into the plant cells as a ready formed enzyme complex. The complex is produced synthetically in the laboratory from the DNA carrying the genetic information to generate the gene scissors and then introduced into the cell nucleus. There the enzyme complex is directly activated and can induce a change at the target site on the chromosome shown in green. The gene scissors are degraded in the cells after a certain length of time.

4. The CRISPR/Cas mode of action

Once the gene scissors are inside the cell nucleus, they use the gRNA to recognize the target site on the genome (see Figure 4). A so-called PAM sequence (protospacer adjacent motif) serves as a stop signal for the enzyme complex. This is a specific DNA sequence and lies adjacent to the target sequence on the genome. The enzyme complex stops at the PAM sequence and uses the gRNA to check whether it matches the DNA sequence in front of it. On the one hand, the gRNA recognizes the target site on the DNA and, on the other hand, binds the cutting component, the Cas protein. If the gRNA and the target site match, the enzyme complex changes its structure, cuts at the target site and mediates a double-strand break of the DNA. The double-strand break is DNA damage and thus an alarm signal for the cell. This signal activates the cell's own repair mechanisms in order to repair the DNA damage as quickly as possible. Thus, the main task of CRISPR/Cas is to cause a double-strand break, everything else is done by the cell itself.

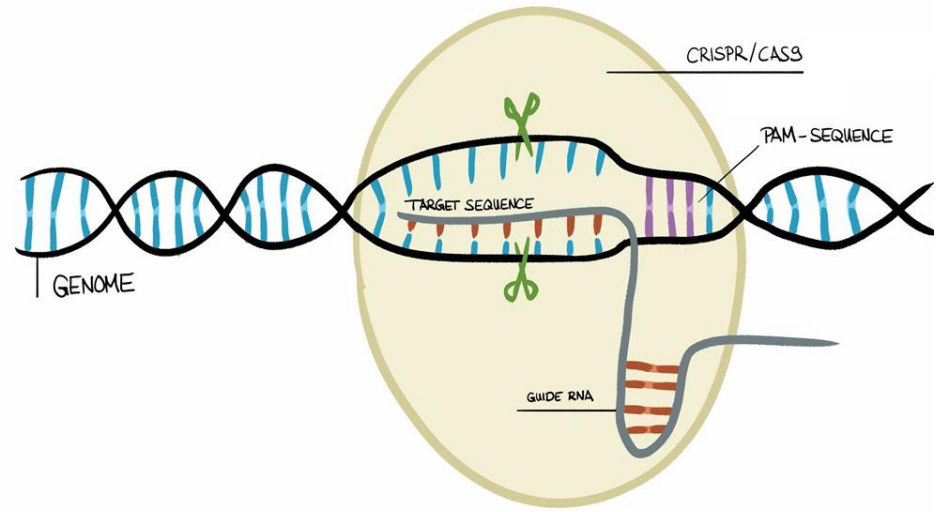

Figure 4: Schematic representation of the CRISPR/Cas mode of action
CRISPR/Cas is guided by a specific gRNA to the target sequence in the genome of the target organism and mediates a double-strand break at this site. The PAM (protospacer adjacent motif) sequence is shown in purple. It acts as the initial recognition sequence for the gene scissors. If the DNA sequence in front of it matches the gRNA, CRISPR/Cas9 is activated and cuts at this site.

After the DNA double-strand break has been mediated by the gene scissors, two different repair mechanisms can be activated. On the one hand, there is a repair mechanism called NHEJ (non-homologous end joining) (see Figure 5), which aims to rejoin the DNA double-strand break as quickly as possible. During that process errors can occur, for example individual bases or smaller DNA segments can be incorporated or smaller areas of the target sequence can be deleted.
Either the original state of the target sequence can be restored or small changes in the DNA sequence can be induced at the target site. If the original state is restored, there is the possibility that CRISPR/Cas will bind again to the target sequence and cut there again. In this

application, the target sequence will most likely be changed in the end [19], which is also the intended effect of this method. This type of application is known as SDN-1 (site directed nuclease-1) and describes small, undirected changes at the target site. This results in a site-specific and undirected repair of the DNA damage.

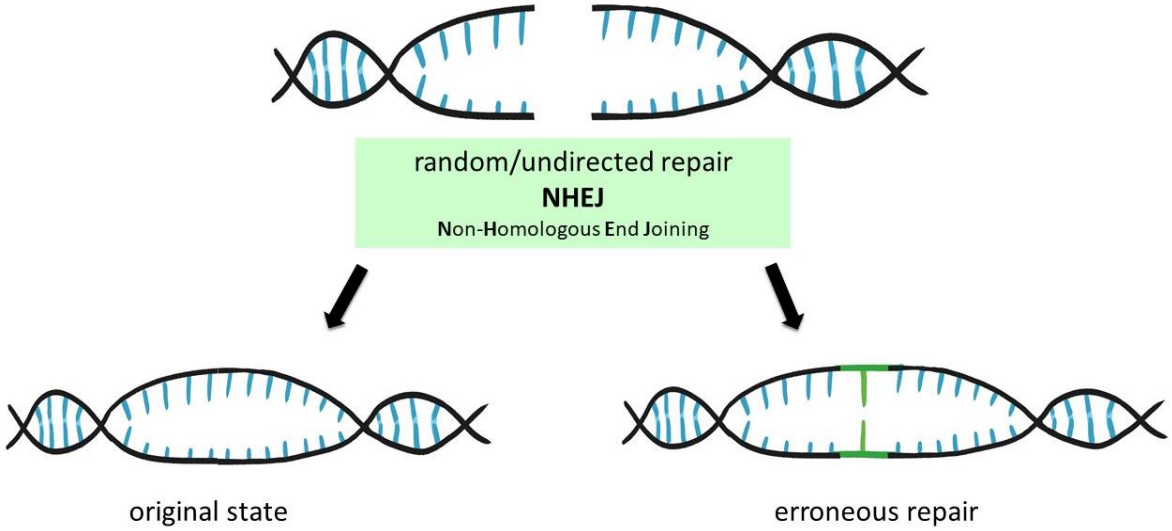

Figure 5: Possible changes induced by SDN-1 applications (site-directed nuclease-1).
The DNA double-strand breaks at the target site caused by the gene scissors activate the cell's own repair mechanisms. With the SDN-1 technique, the NHEJ repair (i.e. non-homologous end joining) either restores the original state or causes small changes at the target sequence of the genome. It is known to be error-prone.

In addition to SDN-1, there are also SDN-2 (site directed nuclease-2) applications, in which the DNA damage is repaired by another repair mechanism, called the HDR system (homology directed repair) (see Figure 6, left). HDR works through homologous recombination, i.e. an exchange of DNA sequences between identical DNA areas. Double-strand breaks in the DNA are repaired naturally using the intact sister chromatid [20]. In SDN-2 applications, DNA templates are synthetically produced in the laboratory and inserted into the cells together with the enzyme complex. The DNA templates are largely identical to the DNA sequence around the target sequence. As a result, the HDR repair is able to incorporate the DNA sequence of the DNA template containing a desired change into the target site. In contrast to SDN-1, in SDN-2 applications the double-strand break generated by the gene scissors is repaired by using a template.

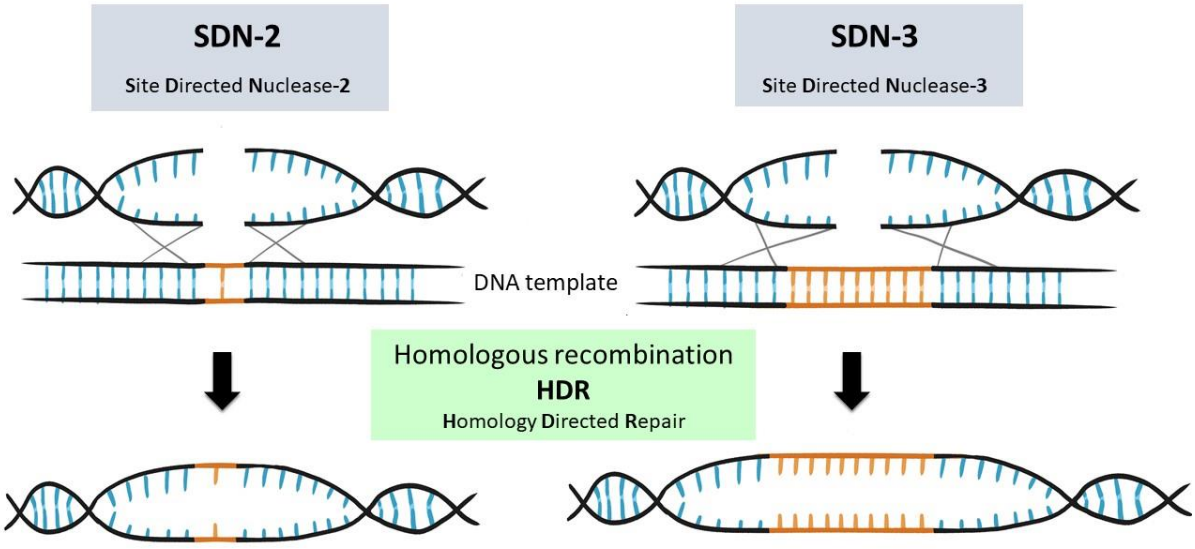

Figure 6: Possible changes induced by SDN-2 applications (site-directed nuclease-2) and SDN-3 applications (site-directed nuclease-3).
In SDN-2 and SDN-3 applications, synthetically produced DNA templates, which are largely identical to the target sequence, are introduced into the cell together with the gene scissors components. A DNA double-strand break activates also the components of the cell's own HDR (homology directed repair) , which leads to the insertion of the DNA template into the target sequence. This technique is used to introduce small targeted changes (SDN-2) or entire genes (SDN-3), shown here in orange, into the target sequence.

Large DNA templates (e.g. containing whole gene segments) can also be inserted at the target site. This type of change is known as a SDN-3 application (site directed nuclease-3) (see Figure 6, right). The difference between SDN-2 and SDN-3 applications is the length of the DNA sequence that is introduced with the DNA template. With SDN-2 only a few base pairs are introduced whereas with SDN-3, longer pieces, e.g. complete genes, can be introduced.

The HDR repair is only active for a relatively short period of time. The NHEJ repair, on the other hand, is almost always active. Most DNA damage is therefore repaired by NHEJ. Compared to animals, HDR repair in plants is also less efficient. The SDN-2 and SDN-3 applications are therefore less efficient than SDN-1. This is clearly reflected in the distribution of applications in plants (see Figure 7). SDN-1 applications are most frequently used, and only a small proportion of studies use SDN-2 or SDN-3 applications [18; 7].

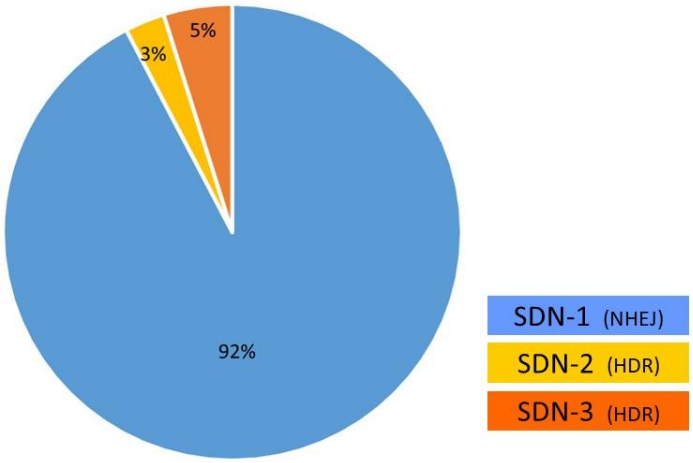

Figure 7: Distribution of SDN applications in plants (derived from [7]).

Between January 1996 and June 2019, 231 market-oriented studies in plants were examined [7]. Most studies use SDN-1 applications (approx. 92%), only a few use SDN-2 (approx. 3%) or SDN-3 (approx. 5%). The most commonly used gene scissor is CRISPR/Cas9 and to a lesser extent TALENs and ZNF, so it must be acknowledged that the majority of the applications in plants originated between 2014 and 2019.

5. Regeneration of plants

Plants can grow their entire life and form new leaves, roots and shoots. Plant cells are therefore able to replace damaged or lost parts. In plant breeding, this property is used to regenerate whole plants from individual plant cells or pieces of tissue with the help of certain nutrition media [21]. Plant cells in which the gene scissors were used, are also regenerated into whole plants by scientists in order to check what changes have been induced at the target sequence. In many species of plants, however, it is still not possible to regenerate whole plants from single cell cultures, which is why the use of gene scissors in such plants is not feasible. At the moment different labs are working on the development of new methods that avoid working with single cells in cell culture. You can find more details on this topic in the background information on the possibilities of CRISPR/Cas.

6. Identification of the intended change

The last step involves identification of the genome-edited plants in which the target sequence has been changed as desired. CRISPR/Cas experiments are carried out in many plant cells. The gene scissors are only introduced into a fraction of them, either as DNA or as a ready formed enzyme complex. The target sequence in each of these individual cells is changed differently. In order to quickly identify the desired editing events, many transformed cells are regenerated into small plants, pieces of their tissues are removed and the genome is sequenced at the target sequence with the aid of PCR methods. This enables the DNA sequence to be

determined and the exact molecular change at the target sequence can be investigated [22; 23]. The plants with the desired changes can be selected at this point.

If at the beginning - as is usually the case - the DNA to form the gene scissors was introduced into the plant cells and integrated into the genome, the DNA must be removed in the selected plants in subsequent crossing steps. This process is called segregation.

What can be done using CRISPR/Cas?

Most often, small changes, i.e. point mutations, short insertions or deletions, are induced at the target site by the gene scissors to switch off one or multiple target genes. The small errors in the DNA can, for example, prevent a target gene from being translated into the corresponding protein. This is also referred to as gene knockout. Scientists can use it to study how a specific gene knockout affects a plant.
In addition, if there are small changes in the sequence, the gene products of the target genes might be changed and their functions altered.

Single or multiple genes can be deleted, i.e. removed from the genome. This is achieved, for example, by using two different gRNAs at the same time. If the gene scissors cut two different target sequences on the same chromosome, the DNA area in between can be deleted. A DNA region with multiple target genes that are to be deleted can therefore simply be “cut out” if the gene scissors cut at the right and left edges of the target area.

In addition, gene expression, i.e. the activity of genes, can be changed in such a way that more, less or no gene product is formed. This can be done, for example, by modifying the regulatory elements of the DNA using the gene scissors. It is also possible to influence gene expression directly by changing epigenetic markers. Epigenetic markers are small appendages to DNA and also to proteins that control gene expression. There are gene scissors applications in which special enzymes are coupled to the gene scissors, which can only change the epigenetic markers on the respective target sequence without cutting the DNA double strand [24].

With the help of the HDR repair and DNA templates, small directed changes in the DNA sequence (via SDN-2 applications) or entire genes can be introduced into the genome in a targeted manner (via SDN-3 applications).

As of May 2021

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